PATENT APPLICATION

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of:

KATHERINE MOLNAR-KIMBER *et. al.*

Appln. No.: **09/576,951**

Filed: **May 24, 2000**

Group Art Unit: **1618**

Examiner: **Ceperly, M.**

For: **ANTI-RAPAMYCIN MONOCLONAL ANTIBODIES**

DECLARATION of PROF. BARRIE W. BYCROFT UNDER 37 C.F.R. § 1.132

Assistant Commissioner
of Patents
Washington, D.C. 20231

Sir:

I, Barrie W. Bycroft, do hereby declare and state:

1 I currently hold the position of Research Professor at the School of Pharmaceutical Sciences at Nottingham University, Nottingham, United Kingdom.

2 I am a medicinal chemist with over 30 years of experience in medicinal pharmaceutical chemistry with a particular emphasis on immunomodulating agents.

3 I graduated from the University of Nottingham with a BSc Honours Degree (Chemistry) in 1960 and a PhD (Chemistry) in 1963. Following a two year NATO Fellowship, which I held at the University of Zurich, Switzerland, I was appointed in 1965 as an Assistant Lecturer in Organic Chemistry at the University of Nottingham. I was subsequently promoted to Lecturer (1967) and Reader (1978). My research interests during this period were predominantly concerned with the chemistry of natural products and, in particular, antibiotics and anti-infective agents. By the end

of the 1970s, my research was increasingly engaging the interface between chemistry and biology. In 1979 I accepted the Chair of Pharmaceutical Chemistry within the then School of Pharmacy at Nottingham to pursue these interests which were predominantly focused in the areas of anti-infective and immunomodulating agents. I was Head of the School from 1985-1988. I was Pro-Vice-Chancellor of the University of Nottingham from 1990-1994.

4 From the early 1980s to the mid 1990s I led a team of chemists and immunologists interested in natural products (derived from microorganisms) with immunomodulating activity, i.e., compounds that suppress or enhance the immune response in some way, and how these compounds influenced the operation of the immune system.

5 In the mid 1990s our studies on immunomodulating agents were largely integrated into the Institute of Infection and Immunity, funded by the Medical Research Council. This followed our discovery of bacterial molecules which down-regulate the immune system by shifting the balance of the T helper cell response.

6 I am familiar with the methodology for the production of antibodies and monoclonal antibodies for use as as pharmacological tools and have been involved in such work in my group's research activities.

7 From 1994-1996, I was Dean of the Graduate School at the University of Nottingham.

8 From 1994-2000 I was the Head of the Pharmacy School and the School of Pharmaceutical Sciences at the University of Nottingham.

9 A copy of my *curriculum vitae* is attached hereto.

10 I am familiar with Claims 33-40 of the above-identified application.

11 I am also familiar with the Office Action dated August 7, 2001 in the above-identified application, wherein the Examiner, *inter alia*, rejects Claims 33-40 as being unpatentable over each of Stella *et. al.*, Failli *et. al.* (A) ('203) and Failli *et. al.* (B) ('307), Kao *et. al.* ('678) and Kao ('477), Caufield and American Home Products in view of each of Sevier *et. al.*, Yelton *et. al.* or Campbell, in further view of Niwa *et. al.* I have reviewed the references relied upon by the Examiner in the Office Action.

12 In my opinion, at the time of the present invention in April 1993, a person of ordinary skill in the art, could not have had a reasonable expectation that one could successfully obtain monoclonal antibodies to rapamycins. This opinion is not changed by the prior art cited by the Examiner which teaches nothing about raising monoclonal antibodies to compounds possessing the

structure and unique biological activity of rapamycins. The detailed reasoning in support of my opinion is set out below.

13 The Examiner refers to literature (Sevier *et. al.*, Yelton *et. al.* and Campbell) which discloses methods for preparing monoclonal antibodies to a variety of antigens, by attaching a hapten carrier to the antigen to form a conjugate and then injecting the conjugate into an animal to activate B cells (which produce antibodies) in the spleen. After two weeks a further booster injection of the conjugate is normally given before the animal is sacrificed and the spleen removed. B cells from the spleen are fused to form hybridomas and appropriate monoclonal antibody producing cells are selected.

14 While these methods had been used successfully to generate monoclonal antibodies to a range of small drugs prior to April 1993, it was known that some small drug conjugates had failed to generate monoclonal antibodies specific to the drug entity within the conjugate. There was little rationale as to which type of drug structures would or would not be recognised by B cells to generate antibodies, other than the general observation that molecules and molecular structures containing regions of conformational flexibility (such as the rapamycin macrolide molecules) were less likely to be recognised by B cells and, therefore, less likely to generate monoclonal antibodies (see, for example, the chapter titled "Structural requirements for Immunogenicity and Antigenicity" by Colin Young p. 1-14 in Molecular Immunology, Eds. M. Zouhair Atassi et al, 1984, in particular, the passages marked on pages 5 and 8, a copy of which is attached hereto). Furthermore, rapamycins are immunosuppressive agents and the generation of monoclonal antibodies to immunosuppressive agents using these methods was known to be difficult and unpredictable. This is because the immunosuppressive action of such drugs disrupts the mechanisms by which antigens are recognised and antibodies produced in the animal. Notably, Sevier *et. al.*, Yelton *et. al.* and Campbell do not exemplify or disclose the generation of monoclonal antibodies to any immunosuppressive agents.

15 As far as I am aware, the only potent immunosuppressive agents specific for T cells in relation to which monoclonal antibodies had sought to be generated prior to April 1993 were cyclosporin and FK506. In both instances, raising the antibodies proved to be problematic. The difficulties experienced in generating monoclonal antibodies to cyclosporin are clearly demonstrated in PCT international application PCT/EP85/00501 in the name of Rosenthaler *et. al.,* which was published on 10 April 1986 (a copy of which is attached hereto). The owners of the patent (Sandoz (now Novartis), a Swiss based pharmaceutical company which is a leader in the field of immunosuppressants for use in transplantation) made numerous unsuccessful attempts to generate monoclonal antibodies to cyclosporin by attaching a carrier protein to the compound. The few monoclonal antibodies Sandoz did raise were found to have relatively low specificity for

cyclosporin, or to be specific with respect to the carrier protein and not to cyclosporin (see, in particular, the paragraph bridging pp. 5-6 of the patent).

16 Monoclonal antibodies against the immunosuppressant, FK506, had been generated with difficulty in the late 1980s, but the procedures were not straightforward. The Niwa *et. al.* application relied upon by the Examiner refers (column 2) to "intensive investigations".

17 Accordingly, by April 1993, with only these two examples to go by, the skilled man knew that it would be difficult and maybe impossible to generate monoclonal antibodies to potent immunosuppressive agents such as the rapamycins. Even if some monoclonal antibodies could be generated using the methods described in Sevier *et. al.*, Yelton *et. al.* and Campbell, there was no reason to predict that they would be specific to rapamycins. Accordingly, at the time of the present invention in April 1993, the generation of monoclonal antibodies specific to rapamycins was totally unpredictable.

18 The Examiner suggests that, given the partial structural similarity between FK506 and natural rapamycin, the fact that monoclonal antibodies to FK506 had been generated, would render it obvious that monoclonal antibodies to natural rapamycin could also be generated. In my opinion, one of ordinary skill in the art would consider this to be incorrect for two reasons. First, while a third of the natural rapamycin molecule may be similar to part of the FK506 molecule, the other two thirds of the natural rapamycin molecule is very different to the remaining portion of the FK506 molecule. I have stated earlier that there was little rationale as to which if any structural features in small molecule conjugates would be recognised by B cells to generate antibodies, other than the general observation that molecules or molecular structures containing regions of conformational flexibility were less likely to be recognised by B cells and, therefore, less likely to generate monoclonal antibodies. The natural rapamycin molecule possesses greater conformational flexibility than FK506. Secondly, and more importantly, in April 1993 it was known that the mode of action (as immunosuppressants) of the rapamycins, both at the cellular and the molecular level, is very different to that of FK506 (and cyclosporin). For these reasons, I, and I believe others of ordinary skill in the field, could not reasonably extrapolate from the experience with FK506 to rapamycins. I will address each of these two points in more detail below.

19 In terms of structure, as illustrated in Figure 1 attached hereto, there are substantial structural differences between natural rapamycin and FK506. Natural rapamycin is a macrolide lactone with a 31 membered ring, which is considerably larger than the 23 membered ring of FK506. Natural rapamycin, unlike FK506, contains within the larger ring structure a triene. Even given the conformational constraints of this triene unit, the degrees of freedom around the additional single bonds within the ring, makes the larger ring structure of rapamycin much more

conformationally flexible than the smaller ring structure of FK506. In addition, the cyclohexyl ring of natural rapamycin is joined to the core macrolide ring by single bonds. Under normal physiological conditions, the dynamic motion of a rapamycin molecule would allow rotation about the single C-C bonds of the flexible arm attaching the cyclohexyl ring, thereby allowing the cyclohexyl ring to occupy a significant space envelope. On the other hand, the cyclohexyl ring of FK506 is joined to the core macrolide ring by a double bond and a single bond, thus severely restricting rotation of the cyclohexyl ring. Accordingly, the side chain of a rapamycin attaching the cyclohexyl ring is significantly more conformationally mobile than the corresponding side chain of FK506. Given these structural and conformational differences, the knowledge that monoclonal antibodies could be generated (with difficulty) to FK506, did not permit the skilled person to be able to predict whether monoclonal antibodies would be able to be generated to rapamycins on the basis of the partial structural similarity to FK506 relied upon by the Examiner. Indeed, given the greater conformational flexibility of the rapamycin molecules, rapamycins would have been considered less likely to be recognised by B cells and therefore less likely to generate antibodies.

20 Furthermore, in terms of biological activity, rapamycins inhibit the immune response in a unique manner. As I shall explain below, it differs from the mode of action of FK506 (a) at the cellular level in terms of rapamycins' effects on both T cells and B cells and (b) at the molecular level in terms of the molecules to which rapamycins bind in order to exhibit their immunosuppressive effects. In order to explain these divergent cellular and molecular activities and their implications for the production of monoclonal antibodies, it is necessary, first, to explain briefly the coordinated mechanisms of the immune response.

21 The key cells controlling the immune response are T lymphocytes (T cells) and B lymphocytes (B cells). Cytokines (soluble signal proteins released by T cells, including the interleukins IL-1 to IL-6) play an essential role in cellular communication and coordinating the immune response. The immune response to a foreign antigen primarily involves (i) cellular responses driven by T cells and (ii) humoral responses i.e. antibody production driven by B cells.

22 The cellular response involves resting T cells recognising presented foreign antigen from an invading pathogen such as a bacteria or virus, and becoming activated and producing cytokines, including IL-2. The IL-2 binds to a receptor which is simultaneously expressed on T cell surfaces. The binding of IL-2 to its cognate receptor results in T cell proliferation affording more T cells (of the T helper type) which in turn produce further cytokines including IL-2. The continuing production of IL-2 thereby maintains the proliferative cascade. Some activated T cells differentiate into cytotoxic T cells which directly attack the invading pathogen. The T

helper cells produce cytokines including IL-4, IL-5 and IL-6 (which stimulate B cells - see below).

23 The humoral response involves B cells recognising and binding to the foreign antigen and, upon receiving further signals from T helper cells via the cytokines (including IL-4, IL-5 and IL-6), becoming activated and differentiating into plasma cells. These plasma cells secrete antibodies that mark the foreign pathogen for destruction by phagocytes.

24 As I said, at the cellular level rapamycins and FK506 have different effects on both T cells and B cells. In relation to the former, by the early 1990s it was well established that FK506 and cyclosporin exhibit their immunosuppressive effects by inhibiting the production of IL-2 (see, for example, Stutz, Transpl. Proc. 24, 4, Suppl. 2, 22-25, 1992, the penultimate paragraph, a copy of which is attached hereto). In the absence of IL-2, T cells cannot proliferate and hence the cellular response generated by T cells is directly inhibited. By contrast, it was well established that rapamycins do not inhibit IL-2 production. Rather, rapamycins act downstream of IL-2 production by blocking the proliferative response of T cells to the signal provided by IL-2. This was a unique mechanism not previously exhibited by any known immunosuppressant in 1993 and it is still unique today. Rapamycins also inhibit the T helper effect for the T dependent production of antibodies by B cells (Luo *et. al.,* Transpl., 53, 1071-1076, 1992, a copy of which is attached hereto).

25 Rapamycins and FK506 also have differential effects on B cells. Although FK506 and cyclosporin inhibit B cell activation when presented with some antigens, they do not directly inhibit antibody production in most models (Stevens *et. al.,* Transpl., 51, 1240-1244, 1991, a copy of which is attached hereto). Indeed, under some circumstances, FK506 has been shown to augment antibody production (Yamamoto *et. al.,* Immunol., 69, 222-227, 1990, a copy of which is attached hereto). On the other hand, rapamycins not only inhibit B cell activation induced by most stimuli through a mechanism clearly distinguishable from that of FK506 and cyclosporin, but also directly suppress antibody production by B cells (Luo *et. al.,* 1992). Accordingly, at the date of the present invention, it was believed by ordinary skilled workers in the field that rapamycins would be considerably more effective in blocking antibody production *in vivo* than FK506 (and cyclosporin).

26 In terms of the molecular basis of the difference in biological activity between FK506 and rapamycins, by April 1993, it was known that, in order to exert its immunosuppressive activity, FK506 binds to a protein (macrophilin) known as FKBP and the FK506/FKBP complex then interferes with the target calcineurin. This important discovery of the molecular target of FK506 had been made by a group at Harvard University in early 1991 (Liu *et. al.* Cell, 66, 807-815, 1991, a copy of which is attached hereto). Liu *et. al.* also teaches that, in order to exert its

immunosuppressive activity, a rapamycin binds to FKBP but the rapamycin/FKBP complex does not interfere with the target calcineurin. Instead, the rapamycin/FKBP complex interferes with a completely different target. Various groups were trying to identify the target of rapamycin around 1993/1994 and the target was subsequently identified and given various names including mTOR, FRAP and RAFT1 (see for example, Brown *et. al.,* Nature 369, 756-758, 1994 and Sabers *et. al.,* J. Biol. Chem. 270: 815-822, 1995, copies of which are attached hereto).

27 The differences in biological activity between rapamycin and FK506 are summed up in Stutz, 1992 (referred to above) where it is stated:

> "*According to our present knowledge, the modes of action of FK506 and CyA* [cyclosporin] *seem to be identical and fundamentally different from that of rapamycin. While FK506 and CyA inhibit the transcription of IL-2 and other lymphokines during the process of T cell activation, rapamycin inhibits the proliferation of these cells induced by, e.g. IL-2. These in vitro effects indicate a common property for the cyclophilin/CyA and macrophilin/FK506 complexes which is not shared by the macrophilin/rapamycin complex. Thus the next step in the chain of intracellular events leading to the specific immunosuppressive effect of CyA and FK506 seems to have been identified, which is clearly separate from rapamycin's mode of action. These specific signalling mechanisms are not restricted to T cells.* [Differences in the effect on the antibody IgE are then reported]."

28 Accordingly, irrespective of any partial structural similarity between FK506 and natural rapamycin, the biological activity of the two molecules both at the cellular and the molecular level was very different. The immunosuppressive action of a rapamycin was likely to disrupt the mechanism by which B cells are activated and antibodies produced (after injection of the rapamycin conjugate into the animal) in a different and unpredictable way compared to that of FK506 (and cyclosporin). In 1993, therefore, the generation of monoclonal antibodies to the potent and unique immunosuppressants, rapamycins, remained problematic and totally unpredictable.

29 The rapamycin conjugate may, itself, not be immunosuppressive (biological activity may possibly be lost due to the large structural and conformational change to a rapamycin molecule caused by the attachment to the carrier molecule). However, it was known in April 1993 that the conjugate was likely to breakdown to the free drug when injected into the animal. According to the present invention, the carrier molecule is attached to a rapamycin molecule by an ester linkage and it was known that following inoculation, such conjugates are susceptible to breakdown to the free drug by non-specific esterases that are distributed in the blood, liver and other organs and tissues of the animal.

30 In the case of small drug conjugates for use in the generation of monoclonal antibodies, there is ample time between injection of the conjugate and sacrifice of the animal for the drug conjugate to be broken down to release substantial quantities of free drug. The release of free rapamycin from the conjugate would block the T cell response as well as the antibody producing ability of the B cells to the conjugate. It was therefore likely that these activities would interfere with the production of antibodies when the rapamycin conjugate was injected into an animal. It was expected in April of 1993 that such interference would most probably prevent the generation of monoclonal antibodies specific to rapamycins.

31 In sum, at the time of the present invention in April 1993:

(a) it was known that some small drug conjugates had failed to generate monoclonal antibodies specific to the drug entity within the conjugate, and there was little rationale as to which structures in small molecule conjugates would be recognised by B cells to generate antibodies, other than a general understanding that molecules possessing regions of conformational mobility (such as the rapamycin macrolide molecules) are less likely to be recognised by B cells, and therefore less likely to generate monoclonal antibodies;

(b) the generation of monoclonal antibodies to potent immunosuppressive agents (such as rapamycins) was known to be difficult and unpredictable;

(c) although monoclonal antibodies had been generated (with some difficulty) to FK506:

(i) there are substantial structural differences between rapamycins and FK506 and rapamycin molecules have significantly greater conformational flexibility than the FK506 molecule; and

(ii) rapamycins exhibit significantly different biological activities to FK506 both at the cellular and the molecular level;

(d) no monoclonal antibodies had been generated to an immunosuppressant that had the same mode of action as rapamycins at the cellular level (i.e. (i) blocked the response of T cells to the IL-2 signal and the T helper effect on B cells; and (ii) suppressed B cell activation and antibody production); and

(e) no monoclonal antibodies had been generated to an immunosuppressant that had the same mode of action as rapamycins at the molecular level (i.e. bound to FKBP and then to a target later identified as mTOR/FRAP/RAFT1).

32 I, and I believe other ordinary skilled people working in the field, could not, therefore, reasonably predict in April of 1993 whether rapamycins' unique inhibitory effects on the immune system,

and in particular rapamycins' effects on B cells and antibody production, would permit the production of monoclonal antibodies upon injection into an animal of a rapamycin conjugate.

Conclusions

In the light of the above, it is evident that at the time of the present invention in April 1993, the ordinary skilled worker in the field would have had no reasonable expectation that one could successfully generate monoclonal antibodies to rapamycins, as claimed in the present application.

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that wilful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code, and that such wilful false statements may jeopardize the validity of this application or any patent issuing thereon.

Date: 7th Feb. 2002 Name: Barrie W Bycroft

Barrie W. Bycroft

SUMMARY

CURRICULUM VITAE

Professor Barrie Walsham Bycroft
BSc, PhD, FRSC, CChem, MRPharmS (Hon)
Date of Birth - 26.01.39

School of Pharmaceutical Sciences, University of Nottingham
University Park, Nottingham NG7 2RD
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Current Position

Professor of Pharmaceutical and Medicinal Chemistry, School of Pharmaceutical Sciences, University of Nottingham (1979 - present).

Previous Positions

Head of School of Pharmacy and School of Pharmacuetical Sciences (1994 - 2000).
Dean of the Graduate School, University of Nottingham (1994 - 1996).
Pro-Vice-Chancellor with special responsibility for research, commerce and industry, and Faculties of Science and Medicine (1990-1994).
Head of the School of Pharmacy and the Department of Pharmaceutical Sciences (1985 - 1989).
Reader in Bio-Organic Chemistry, Department of Chemistry, University of Nottingham (1977 - 1979).
Lecturer in Organic Chemistry, Department of Chemistry, University of Nottingham (1965 - 1977).
NATO Fellow Organisches-chemisches Institut der Universitat Zurich, Switzerland (1963 -1965).

Visiting Fellowship

Visiting Professor, School of Pharmaceutical Sciences, University of Wisconsin, Madison, U.S.A. (1985).
Visiting Professor, Royal Society, Medical School, University of Rio de Janiero, Brazil (1973).
British Council Exchange Fellowship, Germany and Switzerland (1969).

Research Activities

Published over two hundred original papers, review articles and patents at the interface between the biological and molecular sciences (publication list available on request).
These research activities have been funded through SERC, BBSCR, MRC, EU and a range of industrial organisations.

National Committees and Activities

Member of the Scientific Committee and Chair of the New Targets Committee of the Cancer Research Campaign (CRC) (1999 – present).
Chair of the Management Group of the National EPSRC, FTIR Mass Spectrometry Centre, Warwick (1998 – present).
Chair of the Studentship and Fellowships Committee (BBSRC) (1994 - 2000).
Member of the Science and Engineering Board of the Biotechnology and Biological Sciences Research Council (BBSRC) (1993 - 1996).
Vice-Chair of the UK Council for Graduate Education (1993 - 1996).
Chair, Biological Sciences Committee, Science and Engineering Research Council (SERC) (1992 - 1993).
Chair, Royal Society of Chemistry/Biochemical Society Protein and Peptide Group (1990 -1993).
Member of the Food Surveillance Panel of the Ministry of Agriculture Fisheries and Food (1990 - 1993).
Member of the Council of the Biochemical Society (1990 - 1993).
Member of SERC Biological Sciences Committee (1986 -1989).
Chair of the SERC Molecular Recognition Initiative Panel (1989 - 1992). (The largest initiative of the SERC Science Board).
Member of SERC Molecular Recognition Initiative (MRI) Panel (1986 -1989).

International Committees and Activities

Member of the Life Sciences Grant Committee for Enterprise Ireland (Ireland) (1999 - 2001)
Member of the Acciones Intergradas Fellowships and Studentship Exchange Committee (Spain/UK) (1998 - 2000).
Member of the Marie Curie Fellowship Life Sciences Panel. (European Commission) (1994 -1998).
Chair of the European Phytochemical Society (1992 - 1994).

Companies and Other Activities

Director of NOTICE, The University of Nottingham, Commercial Enterprises (1995 -2001).
Director of Biotec Ltd (1997- 2000).
Chair and Director of Cargenex (1997 - 1999).
Director of Screen Science (1996 - 1999).
Non-executive member of Nottingham District Health Authority (1990 - 1994).
Director of Microgen Ltd (1986 - 1989).
Director of Langfield Consultants Ltd (1988 - 1996).

Other Interests

Golf
Tennis
Good Wine and Food
Travel

REFEREED RESEARCH PAPERS

1. **B W Bycroft**, The Structure of Flavasperone (Asperxanthone), *J. Chem. Soc.*, 1962, 40-44 (with T.A. Dobson & J.C. Roberts).

2. **B W Bycroft**, Synthesis of Flaviolin (2,5,7-Trihydroxy-1,4-Naphthaquinone), *J. Chem. Soc.*, 1962, 2063-2064 (with J.C. Roberts).

3. **B W Bycroft**, Synthesis of Flavasperone, *J. Chem. Soc.*, 1963, *929*, 4868-4872 (with J.C. Roberts).

4. **B W Bycroft**, Synthesis of 2,5-Dihydroxy-3-methyl-6-S-butyl-1,4-benzoquinone and its Bearing on the Structure of Nidulin, *J. Chem. Soc.*, 1963, 5748-5151 (with J.A. Knight and J.C. Roberts).

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6. **B W Bycroft**, Synthesis of the Di-*O*-methyl Curvularin Rearrangement Product, *J. Chem. Soc.*, 1964, 2289-2292 (with J.C. Roberts and P.M. Baker).

7. **B W Bycroft**, Ableitung der Absoluten Konfiguration von Alkaloiden der Aspidospermingruppe durch optischen Vergleich mit Alkaloiden der Strychningruppe, *Experientia.*, 1964, *20*, 202, 1-3 (with D. Schumann & H. Schmid).

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12. **W Bycroft**, Ermittlung der Absoluten Konfiguration von Indolinalkaloiden durch Vergleiche der Optischen Rotationsdispersion ihrer $N_{(a)}$-Acylderivate [1], *Helv. Chim. Acta.*, 1966, *49*, 833-841 (with W. Klyne, R.J. Swan & H. Schmid).

13. **B W Bycroft**, Synthesis of (±)-Di-*O*-Methylcurvularin, *J. Chem. Soc.*, 1967, 1913-1915 (with P.M. Baker & J.C. Roberts).

14. **B W Bycroft,** Oxydative Umwandlung von 17-Alkoxy-Aspidospermidin-Derivaten in 17, 17-Dialkoxy-17, 18-dihydro-1,18-dehydro-Aspidospermidine, *Helv. Chim. Acta.*, 1967, *50*, 1193-1201 (with L. Goldman & H. Schmid).

15. **B W Bycroft,** The Structure of Flavomannin, a Metabolite of *Penicillium wortmanni* Klock, *J. Chem. Soc.*, 1968, 2560-2564 (with J. Atherton, J.C. Roberts, P. Roffey & M.E. Wilcox).

16. **B W Bycroft,** Convenient Route to some Naturally Occurring Hydroxynaphthaquinones, *Chem. Comm.*, 1968, 71-72 (with P.M. Baker).

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20. **B W Bycroft,** Synthesis and Stereochemistry of Capreomycidine [α-(2-iminohexahydro-4-pyrimidyl)glycine, *Chem. Comm.*, 1968, 21, 1301-1302 (with D. Cameron, L.R. Croft & A.W. Johnson).

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1992	EP92908821. US5593827	Autoinducers
1993	EP93305221.9	Immunosuppressant Compounds
1994 1995	GB9410143 PCT/.95919498.6	Genes involved in the biosynthetic pathway of carbapenem
1997	GB9725599.6	Control of Biofilm Formation
1997	GB9724859.5	Compounds and their use as Antibacterial Agents

1

Structural Requirements for Immunogenicity and Antigenicity

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I. INTRODUCTION

A. Definitions

Some years ago (Sela, 1969) proposed the following definitions for the main immunological functions of an antigen:

1. Immunogenicity: the capacity to induce an immune response characterized by the formation of specific immunoglobulins and/or specifically committed lymphocytes
2. Antigenicity *sensu stricto*; the capacity of reacting with and binding to specific immunoglobulins and/or cellular receptors
3. Allergenicity: the capacity to elicit various types of allergic reactions and tissue lesions in sensitized animals having specific immunoglobulins and/or committed lymphocytes
4. Tolerogenicity: the capacity to induce specific immunological unresponsiveness, including antibody formation and/or cellular immunity

An understanding of the molecular basis of immunity is partly due to the increase in knowledge over the past 20 years of the number, size, distribution, chemical nature, and specificities of antigenic determinants. A large body of evidence suggests that the induction of humoral and/or cellular immunity depends in part on the molecular structure, and that the structural requirements for immunogenicity differ from those for antigenicity. For example, denatured proteins are often less immunogenic than the corresponding native protein. Also, bacterial proteins are generally better immunogens

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than serum proteins but show little difference in their behavior as antigens, whereas se aggregation of a protein is usually associated with a negligible change in antigenicity b with a considerable increase in immunogenicity.

Knowledge of the sizes and structures of antigenic determinants of polysacchari nucleic acids, and synthetic polypeptides has been obtained from studies of the ability fragments of antigen to inhibit the reaction of the whole molecule with its correspond antibody. Synthetic antigens provide much information for delineating relationships between antigen structure and lymphocyte activation, since it is possible to synthesize tailor-made antigens for asking specific questions. The relationship between structure a antigenicity is more complex for proteins in that it depends to a large extent on the overall three-dimensional configuration of the molecule. Fibrous proteins, however, represent an antigenic situation that could be regarded as intermediate between linear polysaccharides and globular proteins, because of their repetition of amino acid sequer in the polypeptide chain. Studies on the antigenicity of globular proteins are more complex because of the absence of repeating sequences of amino acid residues.

The discussion of antigenicity and immunogenicity in this chapter is divided into three sections. The first section is basically a brief résumé of substances that can act a antigens: proteins, polysaccharides, synthetic polypeptides, nucleic acids, chemically modified antigens, and low-molecular-weight substances. The second section is devoted studies on the immunogenicity of molecules: criteria for immunogenicity and the facto affecting immunogenicity. The third section deals with an analysis of the relationshi between chemical structure, immunogenicity, and lymphocyte function: the hapten-carrier relationship, antigen structural requirements for cell cooperation and lymphocy activation, and structural requirements for the induction of immune responsiveness anc immune suppression. This chapter is not intended to be a detailed discussion encompassing all of the structural requirements for antigenicity and immunogenicity of proteins, polypeptides, polysaccharides, blood group antigens, bacterial antigens, vira antigens, or tumor antigens, since chapters dealing with each of these specifically appea in this book. Rather, this chapter is intended to be more of a general discussion of som of the concepts involved in antigenicity and immunogenicity.

II. ANTIGENS

We should now consider which substances may be antigenic, that is, will induce the formation of antibodies and will then react with these antibodies. Conventionally, a lar amount of information has been derived from: first, investigations using rabbits as the laboratory animal of first choice; second, from the necessity for large-scale immunizatic of humans to prevent various infectious disease; third, from clinical observations of people having diseases, in whom antibody production was taking place.

A. Proteins

Early studies clearly established that proteins were good antigens when injected into an animal species other than the one from which they originated. For example, bovine sen albumin is a good antigen when used to immunize rabbits. When immunizing mammals with homologous proteins, foreignness of protein has conventionally been thought to t a requirement for a protein to be antigenic: that is, the antibody-forming system recognized its own proteins as being not foreign and hence did not react with them. However

recent data with rabbit serum albumin and mouse myoglobin have demonstrated that these self-proteins, when used to immunize a host, can readily elicit an auto-antibody response; see Chap. 2.

B. Polysaccharides

These have proven to be very useful in immunochemical studies because they provide antigens of relatively simple structure by which many of the detailed structural aspects of antigenic determinants and antibody combining sites have been elucidated. Examples of polysaccharides often used as antigens are dextran and levan. Polysaccharide antigens are somewhat unusual in that in the purified form they may stimulate antibody formation in some animal species and not in others; see Chap. 3.

C. Synthetic Polypeptides

These peptides provide a series of antigens of desired specifications for studies on the structural requirements for antigenicity. Chemical methods now allow the preparation of the various types of poly-α-amino acids: first, homopolymers of a single amino acid; second, copolymers in which short peptides of known sequence are linked together; third, random copolymers of several amino acids; fourth, multichain copolymers; fifth, polymers with chains in which a peptide is repeated at intervals.

A very important concept discovered using synthetic polypeptides is that the antigenically important regions of the molecule must be accessible to the antibody-forming system. This was demonstrated as follows: two multichain copolymers were synthesized, each containing lysine, alanine, and a peptide of glutamic acid and tyrosine. Both had a poly-L-lysine backbone. One was designated (T,G)-A--L; in the other the order of addition was reversed (Sela, 1969). A schematic representation of these two synthetic polypeptides is shown in Figure 1.

Only the polymer with the glutamic acid-tyrosine residues on the outside was found to be antigenic in rabbits. If, however, the polypeptide backbone was made as a copolymer

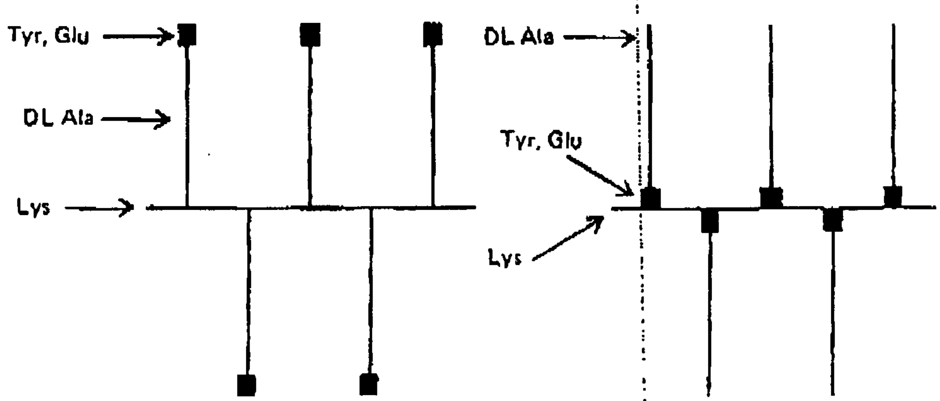

Figure 1 Synthetic diagram of two synthetic copolymers, p(Tyr,Glu)-pDL Ala-pLys and pDLAla-p(Tyr-Glu)-pLys.

of lysine and alanine, so that there was more space between the ε-amino groups of lysine to which the side chains were attached, then a polymer with glutamic acid-tyrosine attached directly to the lysines with alanine side chains at the outside was found to be a strong antigen. Thus accessibility does not require per se a position at the end of the chains.

D. Nucleic Acids

It is very difficult to induce antibody formation to purified nucleic acids. However, it is quite possible to prepare antibodies to DNA, using denatured DNA coupled to methylated bovine serum albumin and injected with adjuvant. Serum antibodies to nucleic acids occur naturally in humans suffering from lupus erythematosus and in NZB/NZW mice. The antibodies in some sera react with denatured DNA, and in others with native DNA, and some antibodies apparently react to antigenic determinants present in both native and denatured DNA. The mechanism by which these antibodies are produced in patients with lupus erythematosus is not as yet fully understood.

E. Chemically Modified Antigens

Much knowledge of the relationships of antigenic determinants came from experiments in which groups of known structure were attached by covalent bonds to protein antigens, after which antibodies were obtained with complementarity directed toward the groups that had been introduced. This work was pioneered by Landsteiner in the 1940s (Landsteiner, 1945), using proteins coupled to low-molecular-weight substances by means of a diazonium salt. The low-molecular-weight groups which are not antigenic in their free form are called *haptens*, and the groups attached to the protein are referred to as the *haptenic group*. The main reactions used routinely in laboratories for introducing such groups into proteins are: iodination, diazotization and coupling, reaction with isocyanates and isothiocyanates, dinitrophenyl (DNP) derivatives, the mixed anhydride reaction, reaction with carbodiimides, reactions with penicillin, and coupling of ribonucleosides and nucleotides to proteins. However, there are certain drawbacks to all of these techniques. First, antibodies may be produced to determinants on the protein which have not reacted with the reagent. Second and more important, the groups introduced may affect the conformation of the protein.

F. Low-Molecular-Weight Substances

Many recent studies have shown that low-molecular-weight substances can function as complete antigens, either in their free form (i.e., not coupled to a large protein) or when adsorbed to carbon particles. Examples of the former are very small synthetic peptides of natural molecules, such as myoglobin and influenza virus. Examples of the latter are hormones such as angiotensin II amide, an octapeptide Asn-Arg-Val-Tyr-Ile-His-Pro-Phe of molecular weight 1045. Also, antibiotics such as bacitracin, gramicidin S, and oxytetracycline are antigenic.

III. IMMUNOGENICITY

A. Criteria for Immunogenicity

Until recently the immunogenicity of an antigen was established by two criteria: the production of specific immunoglobulins and the establishment of cell-mediated immunity.

However, there are some low-molecular-weight antigens, such as DNP-oligolysines, that are nonimmunogenic on their own but are able to induce antibody formation, but not delayed hypersensitivity, when immunized with complete Freund's adjuvant. More recent data have shown that very small free synthetic peptides of natural molecules of 6 to 10 amino acids can induce the formation of antibody, with or without cell-mediated immunity. Since many factors obviously affect the immune response to an antigen, the characterization of a substance as immunogenic or nonimmunogenic can be assigned only in operational terms.

Antibody Formation

Cellular processes involved in antibody formation require the participation of macrophages, bone-marrow-derived lymphocytes (B cells), and thymus-derived lymphocytes (T cells). For many antigens T cells function as helper cells. However, for a small number of antigens, T-helper cells are not required to activate B lymphocytes. These antigens are termed *thymus-independent antigens*. Examples of some thymus-independent antigens are bacterial polysaccharides and some polymerized proteins. The mechanism by which thymus-independent antigens act is not fully understood, but the immune response to such antigens is different from the response to thymus-dependent antigens in that the antibody produced is predominantly of the IgM class and little or no immunological memory is acquired.

The normal course of events in the primary and secondary immune response to most defined antigens is as follows: The first contact with antigen induces an immunological memory and proliferation of antigen-sensitive cells, and a second contact with antigen causes massive differentiation of B cells into antibody-secreting plasma cells. This overall picture for the antibody response is not necessarily the case for all antigens, particularly multideterminant antigens. In multideterminant antigens, antibodies toward the various determinants do not appear all at the same time during the course of immunization (see Chap. 24).

Cellular Immunity

Cell-mediated immunity and delayed-type hypersensitivity is an essential function of T cells. Interaction of specific antigen with antigen-sensitive T cells induces them to proliferate and release soluble mediators responsible for the tissue lesions characteristic of delayed-type hypersensitivity. As yet it is unclear whether the structural determinants on an antigen responsible for lymphocyte proliferation are the same as or different from the structural determinants on the antigen responsible for the molecular mechanisms leading to the release of the lymphocyte mediators. It seems likely that these may well require different molecular properties from the antigen.

B. Factors Affecting Immunogenicity

Size

Traditionally, it has been held that the immunogenicity of antigens is directly related to their molecular size. Very large molecules such as horse spleen ferritin, keyhole limpet hemocyanin, or particulate antigens such as viruses and bacteria are highly immunogenic. On the other hand, polymers of glutamic acid, lysine, and tyrosine elicit the same antibody response even over a threefold variation of molecular weight. However, a

Table 1 Examples of Some Low-Molecular-Weight Immunogens

Immunogen	Molecular weight	Immune response
Artificial and synthetic peptides		
Amino acid copolymers such as GAT, GL, GLΦ	4000–5000	Antibody
Bifunctional haptens	600–1000	Antibody and delayed hypersensitivity
DNP-amino acids	≥300	Antibody and delayed hypersensitivity
Synthetic encephalitogenic peptides	≥700	Delayed hypersensitivity
Synthetic myoglobin peptides	900–3000	Antibody and lymphocyte proliferation
Native peptides		
Insulin and peptide chains	≤6000	Antibody
Gastrin	2000	Antibody
Angiotensin II	1000	Antibody and delayed hypersensitivity
Oxytocin	1007	Antibody

parallel does exist between molecular size and the ability to induce antibody formation for polymerized antigens of various sizes having similar repeating structures. It has been shown that there is a progressive decrease in the immunogenicity and tolerogenicity of fractions of decreasing molecular weight of type III pneumococcal polysaccharide (Howard et al., 1971). However, recently it has been shown that very small peptides, of only 7 to 21 amino acids, of sperm whale myoglobin are very effective both as immunogens and tolerogens (Young and Atassi, 1982a). With levan, on the other hand, a decline in immunogenicity with reduction of molecular weight was not accompanied by loss of tolerogenicity. Generally, high molecular size favors immunogenicity; however there are examples in which large macromolecules are nonimmunogenic, for example high-molecular-weight fully substituted polylysines, polyglutamic acid, and various celluloses.

Generally, low-molecular-weight antigens are not the best immunogens. As shown in Table 1, several compounds below 5000 molecular weight have been demonstrated to be immunogenic. One important question to ask in this context is: What is the minimum molecular size for immunogenicity? Using a series of oligolysines of increasing sizes monosubstituted on the α-N-terminal lysine by a DNP group, Schlossman (Schlossman et al., 1965) showed very clearly that mono-α-DNP-oligolysines smaller than heptalysine were incapable of eliciting anti-DNP-antibody production or cellular hypersensitivity. However, exceptions to this heptapeptide rule are the p-azobenzenearsonate derivative of tyrosine and histidine and dinitrophenylated amino acids. Using free synthetic peptides of increasing size, it has recently been shown that a free antigenic site (i.e., uncoupled) of as few as six amino acids is effective in eliciting both humoral and cellular immunity (Young and Atassi, 1982b).

The experiments with DNP-oligolysines of varying length established that delayed-type hypersensitivity requires a molecule of at least seven amino acid residues, whereas smaller substituted oligolysines were capable of inducing antibody formation. However, this finding occurred only when the immunogens were injected together with complete Freund's adjuvant containing mycobacterium. However, without adjuvant a reverse phenomenon occurs; that is, the smaller the molecule, the more it tends to induce delayed-type hypersensitivity in the absence of circulating antibody. It is possible that these smaller molecules containing a single immunodeterminant may preferentially induce delayed-type hypersensitivity over humoral immunity. In general, weak immunogens favor the establishment of the delayed-hypersensitivity state.

Conformation

For large antigens the antigenic determinants are those portions which are superficially exposed or protruding parts of the antigenic molecule. For example, the antigenic sites of sperm whale myoglobin have been shown (Atassi, 1975) to reside at conformationally sensitive portions of the polypeptide chain, located on the surface of the molecule and sometimes referred to as the "elbow regions." For multichain amino acid copolymers it has been demonstrated that the immune response can be directed against conformational determinants produced by both the tertiary and quarternary structures. For example, the tripeptide Tyr-Ala-Glu attached to a branched polymer of DL-alanine and lysine induces antibodies inhibitable by the tripeptide, whereas a high-molecular-weight polymer of the tripeptide forms α helices and induces antibodies against conformational determinants not present in the tripeptide.

It has been assumed that an α-helical configuration favors immunogenicity, and that oligopeptides should only be immunogenic when large enough to assume the α-helical conformation. Oligopeptides derived from L-alanine and co-oligomers of L-alanine with γ-methyl-L-glutamate possess a random coil conformation in trifluoroacetic and dichloroacetic acids, whereas in trifluoroethanol the pentamer and larger oligomers are in helical form. The lowest polymeric size for an α helix in nonaqueous solvents is an octapeptide of γ-methyl glutamate nonalanine or undecapeptide of β-methylaspartate. However, studies on the immunogenicity of oligopeptides requires more knowledge not only of their primary structure but also of their stereochemical conformation.

Antigenic determinants of globular proteins have been designated to be either of a continuous or a discontinuous type. Continuous antigenic determinants are those formed by a linear sequence of amino acids of a polypeptide chain; for example, antigenic site 1 of sperm whale myoglobin occurs in amino acid sequence 15-21. Discontinuous antigenic determinants are those formed by amino acid residues which are contiguous but not part of a linear amino acid sequence, for example the antigenic sites of lysozyme. A much more detailed discussion of these continuous and discontinuous antigenic sites appears in Chapter 2.

Effect of alteration of conformation on antigenicity. It has been known since the 1940s that denaturation due to heating or to chemical modification results in a reduction of immunogenicity and an alteration in antigenicity. The structural requirements for antigenicity and immunogenicity have since been examined in much more detail using various well-characterized procedures.

Cleavage of all the intramolecular disulfide bonds in proteins, destroying non-covalent interactions and causing unfolding of polypeptide chains, has been shown to

result in a large reduction in activity of antibodies elicited to the native protein, for example ribonuclease, papain, trypsin, lysozyme, and bovine serum albumin. However, when only two of the four disulfide bonds of ribonuclease are cleaved, the partially reduced protein is immunologically indistinguishable from native ribonuclease. This result, taken together with results from other partially reduced proteins, suggests that disulfide bonds per se do not play a major role in determining antigenicity. The secondary role of disulfide bonds in determining antigenicity is probably due to the fact that disulfide bridges do not direct the folding of the polypeptide chain but stabilize the most thermodynamically stable conformation.

Because of the close relationship between conformation and antigenicity, it would be expected that a small conformational change would result in a change in antigenic reactivity. One of the best characterized systems demonstrating this is the removal of the heme group from sperm whale myoglobin. The loss of the heme group in apomyoglobin is associated with a decrease in helical content of the protein and an increase in asymmetry of the protein; that is, the folded polypeptide chain of apomyoglobin is more flexible. Differences between the antigenic reactivities of metmyoglobin (heme group present) and apomyoglobin have been clearly shown (Crumpton and Wilkinson, 1965). Figure 2 shows that metmyoglobin forms, at equivalence, larger amounts of precipitate than that formed by apomyoglobin, with antisera to metmyoglobin.

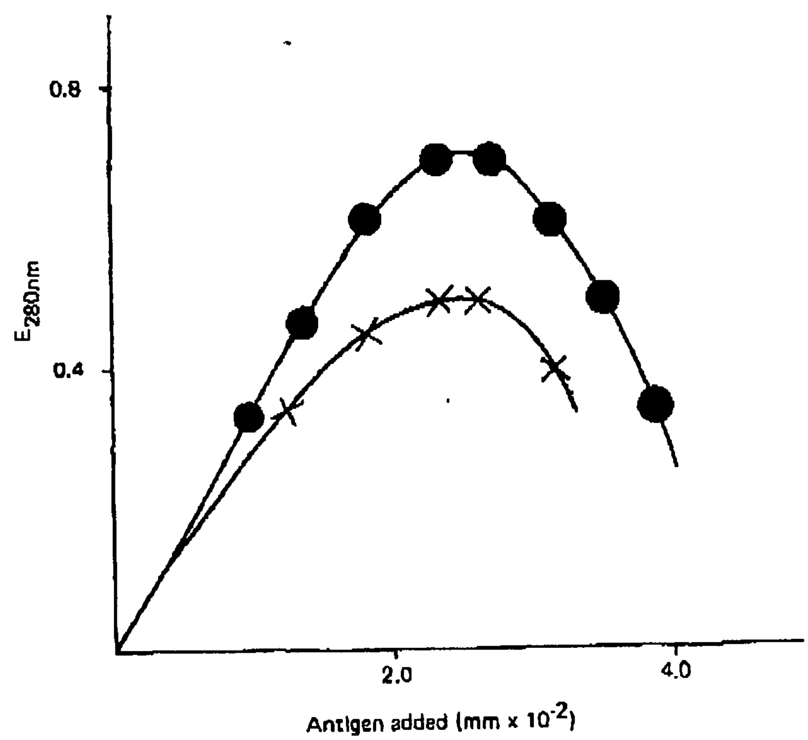

Figure 2 Amounts of precipitate formed by the addition of sperm whale metmyoglobin (●– ···●) and of apomyoglobin (X ——X) to an antimetmyoglobin serum.

Electrical Charge

There are two aspects of electric charge which should be discussed. First, is an electrical charge on the immunogen required for eliciting an immune response? Second, does a net electrical charge on the immunogen affect the characteristics of immunoglobulins evoked by the immunogens?

A net electrical charge per se on the immunogen does not seem to be required, since nonionizable synthetic polypeptide antigens, soluble in water and nonionizable at neutral pH, are immunogenic. However, basic peptides and basic proteins with clusters of positively charged amino groups are very good immunogens, for example the basic protein of myelin. High electrical basic or acidic net charge may secondarily enhance immunogenicity by the formation of complexes or aggregates with the adjuvant used in immunization. Since lymphocyte and macrophage membranes are negatively charged, it could be argued that positively charged antigens are attracted nonspecifically to their membranes, thereby facilitating antigen presentation.

It has been clearly shown that there is an inverse relationship between the net electrical charges of immunogens and the immunoglobulins elicited by them. This has been demonstrated very clearly with antibodies to acidic diptheria toxoid and to negatively charged synthetic amino acid copolymers. Interestingly, antibodies produced to p-azobenzene-arsonate groups bound to basic poly-L-lysyl ribonuclease or to acidic rabbit serum albumin showed that the antibody charge was the opposite of the net charge of the molecule, rather than charges within limited areas of the azobenzenearsonate determinant. For large globular proteins it is possible that charged residues may contribute strongly to the specificity of antigenically reactive determinants (see also Chap. 16).

Adjuvants

Many substances, such as oil emulsions and aluminum hydroxide, have been described as enhancing immunogenicity. There are various effects of adjuvants that should be emphasized in this context: first, enhancing the immunogenicity of a substance that is immunogenic when immunized into an animal on its own; second, conferring immunogenicity to substances that are not immunogenic when immunized into animals on their own; third, modifying the type of immune response of an immunogen (e.g., humoral immunity or delayed-type hypersensitivity). The enhancing effect of adjuvants, such as Freund's complete or incomplete adjuvant, has been well demonstrated for macromolecular and polypeptide antigens. However, not all antigens are equally sensitive to the effects of adjuvants; for example, polysaccharide antigens show no enhanced immunogenicity when immunized in complete Freund's adjuvant. For macromolecular antigens, complete Freund's adjuvant and mycobacteria have been shown to shift the type of immune response elicited; for example, in guinea pigs complete Freund's adjuvant increased the production of γ_2 immunoglobulin and enhances delayed-type hypersensitivity.

Various adjuvants contain mycobacteria or *Bordetella pertussis*, which stimulate macrophage activity, and it is possible that the increased digestion of immunogens by macrophages could result in the formation of active peptides containing single antigenic sites and thereby activating antigen-sensitive lymphocytes. On the other hand, inert polystyrene latex particles of about the same size and hydrophobicity as mycobacteria also are excellent adjuvants, at least for the elicitation in rabbits of antibody formation to human IgG (van Oss et al., 1976). It thus may be the simple hydrophobicity of such particles that favors the adsorption of the antigen and allows its presentation in a regular array on the surface of insoluble particles.

Dose of Antigen and Route of Administration

Both of these factors can play a considerable role in the development of an immune response and the type of response obtained. These factors are of great importance for both high- and low-molecular-weight antigens, since the former can contain very low molar concentrations of single antigenic sites which can function as very low molecular weight antigens. One very good example to quote here is the response to the synthetic octapeptide angiotensin II: injection in complete Freund's adjuvant results in delayed-type hypersensitivity, whereas intradermal immunization evokes antibody production. When investigating the immunogenicity of high- or low-molecular-weight antigens, one should carry out an examination of the effects of different routes of immunization as w as complete dose-response curves for each route of immunization. Thus the definition of substance as immunogenic or nonimmunogenic can be accepted only in operational term

Genetic Factors

Genetic factors in the immune response, specifically the immune response (Ir) genes, ha been reviewed extensively (McDevitt and Landy, 1972). Information clearly defining an immune response gene to synthetic polypeptides came from the study of (T,G)-A--L (see Fig. 1), which bears a restricted number of antigenic determinants. The antigen (H,G)-A--L is a related polypeptide in which the tyrosine residue has been substituted by a histidine residue in the antigenic determinant. Studies with (T,G)-A--L and (H,G)-A--L have clearly emphasized the importance of genetic background of the anim species used in defining the immunogenicity of these substances. The CBA strain of mouse is a low responder to (T,G)-A--L and a high responder to (H,G)-A--L, whereas the C57 strain of mouse is a high responder to (T,G)-A--L and a low responder to (H,G)-A--L. It was shown that the immune response to each of these antigens is under separate Ir-gene control. Since this initial demonstration of the genetic basis of the immune response, many antigens have been shown to be under genetic control, not only in mice, but also in guinea pigs, rats, chickens, monkeys, and humans. Examples of some of the many antigens the response to which has been shown to be under genetic control are: lactate dehydrogenase, ovalbumin, ovomucoid, ribonuclease, staphylococcal nuclease, bovine gamma globulin, and myoglobin.

Studies on the genetic control of the immune response to complex antigens, such a sperm whale myoglobin, have revealed some very interesting features concerning the structural recognition of antigenic portions of the molecule. For example, it has been shown that each of the five individual antigenic sites in myoglobin are controlled by unique, distinct Ir genes. Furthermore, strains of mice which are high responders to myoglobin do not necessarily respond to the same antigenic sites of the molecule. Also, the contribution that the dose of antigen used in immunization plays in the genetic recognition of antigenic sites has been clearly demonstrated. For example, strains of mic which are nonresponders to myoglobin or any of its antigenic sites at an immunizing dos of 50 μg will respond well to myoglobin and all of its antigenic sites at higher immunizing doses (see also Chap. 25).

IV. ANALYSIS OF RELATIONSHIP BETWEEN CHEMICAL STRUCTURE, IMMUNOGENICITY, AND LYMPHOCYTE FUNCTION

A. Hapten-Carrier Relationship

Much of the understanding of the nature and specificity of antigen-antibody reactions results from the early pioneering work of Landsteiner, using small substances which are

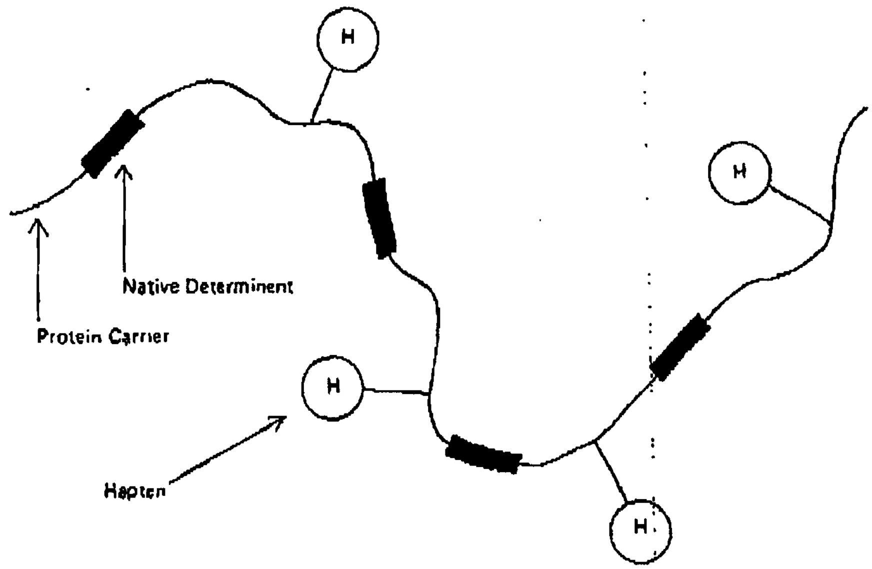

Figure 3 Illustration of hapten-protein conjugate.

not immunogenic but can react with antibodies of appropriate specificity. In order to elicit antibodies to the haptenic determinant, the hapten is first coupled to a protein. The conjugated proteins elicit antibodies specific for the hapten, as demonstrated by the capacity of the free hapten to bind antibody. The protein, called the carrier, has its own set of native determinants as well as the new determinants introduced by the conjugated hapten, as represented in Fig. 3.

It has been known for some time that the production of antibodies against the haptenic determinant involves a structural configuration of the carrier molecule. The importance of the carrier moiety is demonstrated by the finding that animals that cannot respond by antibody production to the hapten DNP when carried on a nonimmunogenic carrier can respond when the DNP is bound to an immunogenic carrier. It thus appeared that a recognition of antigenic determinants on the carrier molecule was a prerequisite for the induction of antibody as well as for recognition of the haptenic determinant. The work of Mitchison (1971) has clearly shown that different lymphoid cells respond to carrier and hapten determinants and that both responses are required for the production of antihapten antibody. Experiments in mice have clearly demonstrated that the carrier-sensitive cells are T lymphocytes (see Chap. 24).

The insertion of spacer molecules between the hapten and carrier determinants on the conjugate does not influence the immunogenicity of the conjugate, indicating that the hapten and carrier portions are not acting as part of a single determinant. The separation of the carrier determinant and haptenic determinant of bifunctional molecules with spacers of varying size has allowed an assessment of the spatial requirements between hapten and carrier for an antihapten response. Using the hapten DNP, the carrier L-tyrosine-p-azobenzenearsonate (RAT), and the spacer 6-aminocaproic acid it has been established that cooperation can be implemented by an antigen in which the hapten and carrier moieties were separated by less than 8 Å.

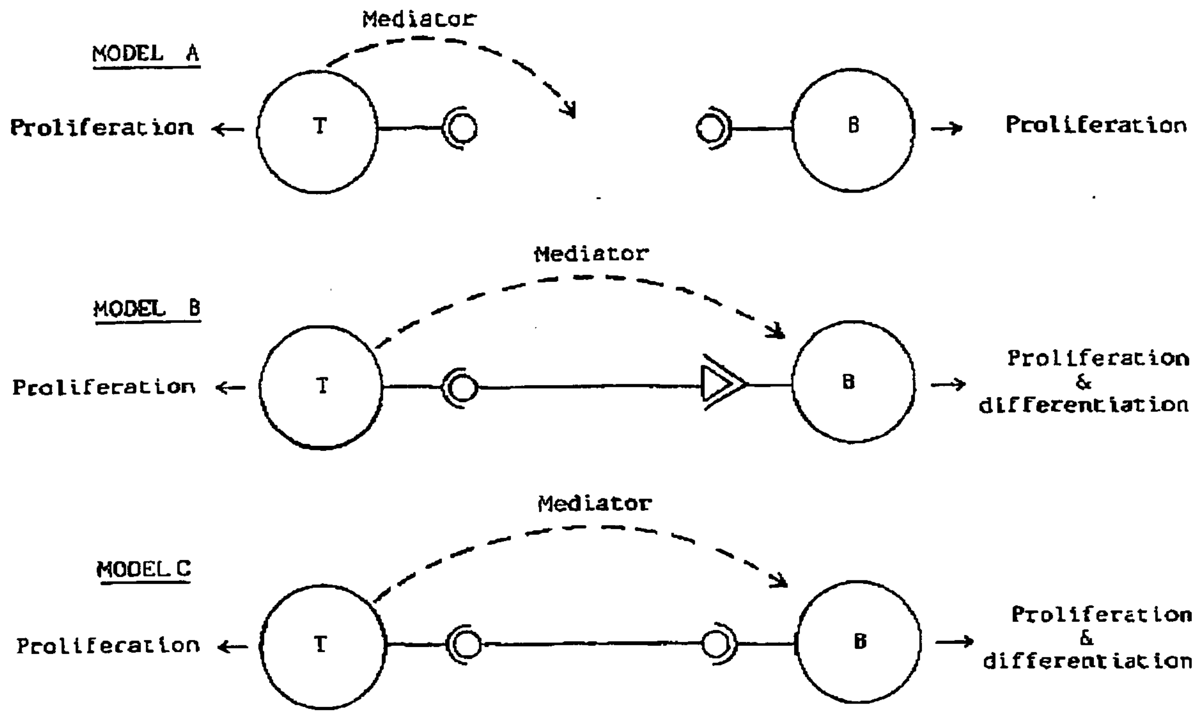

Figure 4 Various models of cooperation between T and B cells in response to: A, a monofunctional antigen containing one immunogenic determinant only; B, an asymmetric bifunctional antigen containing one immunogenic and one haptenic determinant only; C, symmetrical bifunctional antigen containing two immunogenic determinants. O, Immunogenic determinant; Δ, haptenic determinant.

However, there are certain exceptions to the hapten-carrier concept. For example, some polymeric antigens composed of identical repeating units such as endotoxin lipopolysaccharides, pneumococcal polysaccharides, and polymerized flagellin do not require the intervention of T cells and of carrier-specific cells. Also, recent work has shown that uncoupled peptides of macromolecules of only six amino acids and containing only one antigenic determinant can elicit antibody production when immunized with complete Freund's adjuvant. However, most multivalent hapten-protein conjugates show a strong carrier specificity in the anamnestic antibody response.

B. Structural Requirements of Antigens for Cell Cooperation and Lymphocyte Activation

Cell cooperation mediated by identical determinants on an antigen molecule has been investigated using a series of bifunctional antigens with different spacers (Goodman et al., 1974). Using symmetrical bifunctional antigens it was found that there was a good correlation between helper activity and the steric availability of determinants, thus strengthening the concept that at least two determinants, which may be identical, must be accessible to elicit antibody response. This finding also strengthens the thesis of antigen-bridging models of cell cooperation, which are summarized in Fig. 4.

This same series of bifunctional antigens was also used for comparing the efficiency of single-point and two-point antigen binding in triggering lymphocyte proliferation. Using these antigens it was demonstrated that rigidly spaced bifunctional antigens are poor stimulators of antigen-induced proliferation, whereas they served very well for cell cooperation. Thus the structural requirements for cell triggering and for cell cooperation differ markedly.

C. Structural Requirements for Induction of Immune Responsiveness and Immune Suppression

A particularly good example to demonstrate these structural requirements is the L-tyrosine-p-azobenzenearsonate (RAT) immunogenic system. Two series of RAT analogues were synthesized, one in which substitutions were made at the arsonate position and the other in which the side chain of tyrosine was modified. It was found that

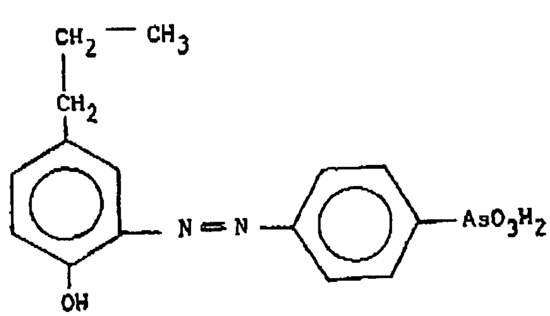

Figure 5 (p-Hydroxyphenyl)propane-azobenzene-p'-arsonate (RAN).

other charged moieties could substitute for arsonate without loss of immunogenicity. On the other hand, removal of both charged groups from the tyrosyl side chain completely abolished immunogenicity. This nonimmunogenic analogue of RAT with a propyl side chain was designated (p-hydroxyphenyl)–propane-azobenzene-p′-arsonate or RAN (see Fig. 5).

Since RAN is a very close analogue of RAT, and lacks perceptible immunogenicity, it has been used to look at the structural requirements for tolerogenicity. RAN, as well as RAT, induces specific unresponsiveness to RAT when immunized with incomplete but not with complete Freund's adjuvant, as measured by delayed hypersensitivity and antigen-induced DNA synthesis. Thus a compound without perceptible immunogenicity is capable of rendering T cells unresponsive. Although not clearly proven yet, it seems likely that this phenomenon is mediated by suppressor T cells.

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Immunosuppressive Macrolides

A. Stütz

IN contrast to the cyclic peptide, Sandimmune [cyclosporine (CyA)], FK 506 is a macrolide lactone containing a number of highly complex functional groups incorporated in a 23-membered ring (Fig 1).

FK 506

How was FK 506 discovered and profiled? As a consequence of the discovery of Sandimmune, researchers from the Fujisawa Co started to look for novel immunosuppressants in cultured broths of microorganisms, isolated from soil samples. The discovery of FK 506 started in 1982 with a soil sample from Tsukuba. This organism differed from previously described *Streptomyces* on the basis of its morphologic, cultural, and physiologic characteristics and has been designated *Streptomyces tsukubaiensis*. In 1984, the first fermentation, purification, and isolation of FK 506 were performed and immunosuppressive effects in mice were discovered. X-ray analysis and the elucidation of its chemical structure came in 1985 in addition to the first studies on immunosuppressive effects in vitro and in experimental transplantation models. One year later the first biological data were reported at the Eleventh International Congress of the Transplantation Society in Helsinki.[1] The first use of FK 506 for liver, kidney, and pancreas transplantation was published in 1989[2] by Starzl, followed by a series of presentations at the Fourth Meeting of the European Society for Organ Transplantation, Barcelona[3] (Table 1).

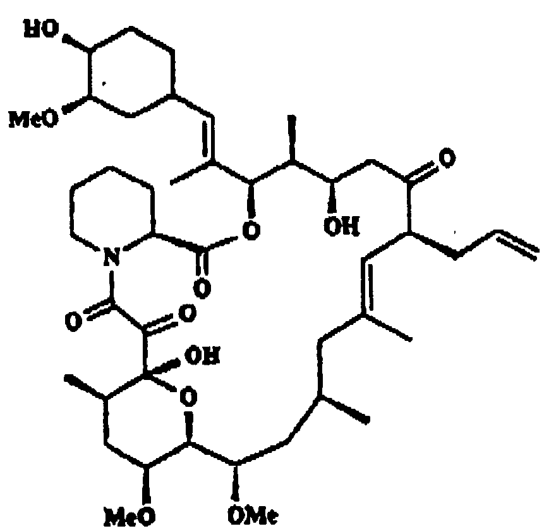

Fig 1. FK 506.

Table 1. Discovery of FK 506

1982–1983	T Goto, T Kino: Screening of specific immunosuppressants from fermented broths
1984	M Iwami, T Kino: Isolation and taxonomy of *Streptomyces tsukubaensis*
	M Matanaka, T Kino: Fermentation, purification, and isolation of FR900506 (FK 506)
	T Kino, N Inamura: Discovery of immunosuppressive effects in mice (DTH, PFC, and GVH)
1985	A Kuroda et al and T Taga et al: Determination of the structure of FK 506 (chemical degradation and x-ray analysis)
	T Goto, T Kino: Immunosuppressive effects of FK 506 in vitro
	T Ochiai et al and N Inamura et al: Discovery of immunosuppressive effect of FK 506 in experimental transplantations (skin, heart, and kidney)
1986	T Ochiai et al: Publication of immunosuppressive activities of FK 506 in vivo and in vitro
1989	TE Starzl et al: Use of FK 506 for liver, kidney, and pancreas transplantation

RAPAMYCIN

Rapamycin is a macrolide antibiotic like FK 506, and shares with it many structural features (Fig 2).

This compound has been known since 1975, when its structure and antifungal properties were reported. Although its immunosuppressive activity was already known in 1977, its potential in transplantation was not realized until Calne reported the first data in 1989 (Table 2).

FK 506 AND RAPAMYCIN ARE DISSIMILAR IN EXPERIMENTAL MODELS OF ALLERGIC CONTACT DERMATITIS

Both FK 506 and rapamycin have been reported to have comparable immunosuppressive potency in vitro and in vivo.[4] Both compounds inhibit T-cell activation, but by different modes of action.[3,4] Based on this information,

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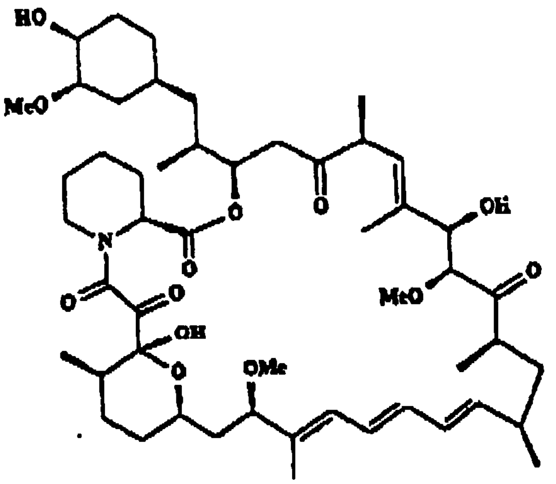

Fig 2. Rapamycin.

Table 3. Animals and Methodologies Used in Allergic Contact Dermatitis Models

Species:	Mouse	Guinea pig
Allergen:	Oxazolone, 2%	DNFB, 0.5%
Test site:	Pinnae [2]	Lateral abdomen [2]
Treatment schedule:	1 × 30 min PC	2 × (30 min and 6 h PC)
Evaluation (24 h PC)	Pinnal weight	Skin thickness and skin color
Species:	Domestic pig	Cynomolgus monkey
Allergen:	DNFB, 1%	DNFB, 0.5%
Test site:	Dorsolateral back [24]	Trunk [8]
Treatment schedule:	2 × (30 min and 6 h PC)	1 × 30 min PC
Evaluation (24 h PC)	Gross lesion and skin color	Gross lesion and microvascular perfusion

PC: post challenge; figures in brackets indicate number of test sites per animal.

and assuming that T cells play a major role in allergic contact dermatitis and other inflammatory/hyperproliferative skin diseases, we performed a comparative study on the topical efficacy of FK 506, rapamycin, CyA, and the corticosteroids dexamethasone and clobetasol in animal models of allergic contact dermatitis.[7,8]

The animals used in this study were mice, guinea pigs, domestic pigs, and cynomolgus monkeys. The allergens were oxazolone in mice and DNFB in the other animals. Particular emphasis was given to the pig model, which has not been reported or used in previous pharmacologic studies. Pigs have been used, because their skin is closely related to human skin.

After sensitization, the challenge was performed on day 12 on the dorsolateral back of the animals, and the test sites were treated twice (30 minutes and 6 hours after challenge) with drug vehicle or active compound preparation. Evaluation of the treatment-related effects was performed at the peak of the inflammatory response (24 hours after challenge). Each test site was evaluated visually for gross changes (ie, intensity and expanse of erythema and induration). Skin color was determined with a reflective color measurement. Microvascular perfusion was measured with a Periflux Laser Doppler Perfusion Monitor (Table 3).

In the pig model, the standard corticosteroids, dexamethasone and clobetasol, were active according to their relative potency. FK 506 demonstrated dose-dependent activity and proved more potent than even clobetasol. In contrast to FK 506, the structurally related rapamycin turned out to be topically ineffective in the pig model, although both compounds have been reported to be immunosuppressants of comparable potency both in vitro and in vivo (Fig 3).

Furthermore, unlike CyA, topical rapamycin did not inhibit contact hypersensitivity-induced pinnal swelling of mice. Here again, FK 506 was highly potent at concentrations as low as 0.01% (Table 4). Since FK 506 also showed efficacy in cynomolgus monkeys, these studies give evidence that topical application of FK 506-type compounds might have therapeutic potential in skin diseases. In addition, these findings raise the issue as to what mechanisms are involved in contact hypersensitivity-induced inflamma-

Table 2. Discovery of Rapamycin

1975	Antifungal activity, structure—SN Sehgal et al: *J Antibiot* 28: 727
1977	Immunosuppressive activity in vitro—RR Martel et al: *Can J Physiol Pharm* 55:48
1989	Use in organ allografting—RY Calne et al: *Lancet* II, 227

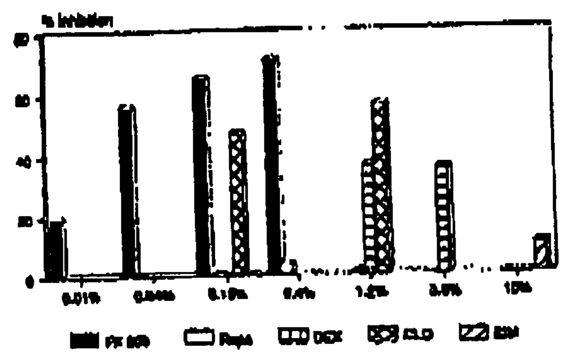

Fig 3. Inhibition of gross changes of allergic contact dermatitis (domestic pig).

Table 4. Treatment-Related Inhibition of Allergic Contact Dermatitis

Model	Drug Concentration (%)	FK 506	Rapamycin	CyA	Dexamethasone
Mouse	0.13	74	0	51	78
	0.04	77	21	36	53
	0.01	55	21	11	41
Guinea pig	0.13	NT	NT	44/30*	44/59*
	0.01	56/61*	NT	NT	NT
	0.04	NT	NT	18/14*	17/9*
	0.004	43/42*	NT	NT	NT
Domestic pig	10.0	NT	NT	6/0	NT
	3.6	NT	NT	NT	37/32†
	1.2	NT	0/0†	NT	42/34†
	0.4	71/77†	4/2†	NT	NT
	0.13	65/67†	NT	NT	NT
	0.04	56/51†	NT	NT	NT
	0.01	19/31†	NT	NT	NT
Nonhuman primate	0.13	56/62‡	NT	NT	NT

*X/Y: Percent inhibition of skin thickening/inhibition of skin reddening.
†X/Y: Percent inhibition of gross changes/inhibition of skin reddening.
‡X/Y: Percent inhibition of gross changes/inhibition of microvascular blood flow.
NT: not tested.

tion, and how FK 506 (but not rapamycin) interferes with these processes.

INTRACELLULAR RECEPTORS OF FK 506, RAPAMYCIN, AND CyA

CyA binds with high affinity to an intracellular receptor protein, cyclophilin, a major cytosolic constituent of all cells. Macrophilin (FKBP), an additional type of intracellular receptor protein, has been recently identified, and shows no structural homology with cyclophilin. Macrophilin and cyclophilin can catalyze the isomerization of the cis and trans form of peptidyl-prolyl amide bonds and are, therefore, called rotamases (Fig 4). Both FK 506 and rapamycin bind to macrophilin, but not to cyclophilin.[9]

When comparing the chemical structures of FK 506 and rapamycin, the left parts of both molecules are almost identical. This common partial structure constitutes their binding domain to macrophilin. As has been demonstrated recently, a partial structure containing the binding domain binds to macrophilin, but has no immunosuppressive effect.[10] As a consequence, the binding domain is necessary, but not sufficient for immunosuppression. The right part of both FK 506 and rapamycin, the effector domain, must be involved in the process leading to inhibition of T-cell activation.

In 1991, the solution structure of human macrophilin, as well as the X-ray studies of the complexes macrophilin-FK 506 and macrophilin-rapamycin were published by Schreiber.[11,12] These studies proved that FK 506 and rapamycin bind in the cavity that serves as the rotamase-active site.

In the complex, the pipecolinic ring system of the binding domain mimics the proline part of the substrates for the enzyme macrophilin. In the unbound form, the pipecolinic ring in FK 506 is oriented in the *cis* configuration, whereas in the bound form this substructure is in the *trans* configuration. In rapamycin, the corresponding pipecolinic part is *trans*-oriented both in the bound and unbound form. Thus, in the bound form, the conformations of FK 506 and rapamycin are identical, which supports a common mode of drug binding.

According to our present knowledge, the modes of action of FK 506 and CyA seem to be identical and fundamentally different from that of rapamycin. While FK 506 and CyA inhibit the transcription of IL-2 and other lymphokines during the process of T-cell activation, rapamycin inhibits the proliferation of these cells induced by, eg, IL-2. These in vitro effects indicate a common property for the cyclophilin/CyA and macrophilin/FK 506 complexes which is not shared by the macrophilin/rapamycin complex. Indeed, calcineurin has been revealed recently[13] to be a common target of the cyclophilin/CyA and macrophilin/FK 506 complexes, but not of the macrophilin/rapamycin complex. Thus, the next step in the chain of intracellular events leading to the specific immunosuppressive effects of CyA and FK 506 seems to have been identified, which is clearly separate from rapamycin's mode of action. These specific signalling mechanisms are not restricted to T cells. Schreiber[14] reported that CyA and FK 506, but not rapamycin, inhibit IgE receptor-mediated exocytosis of secretory granules from the rat basophilic leukemia cell line, RBL-2H3.

The understanding of the molecular mechanisms involved in immunoregulation has dramatically increased within the past few years. In this context, CyA and the macrolides, FK 506 and rapamycin, have played and will continue to play key roles as pharmacologic probes[4] of the immune system and signal transduction pathways.

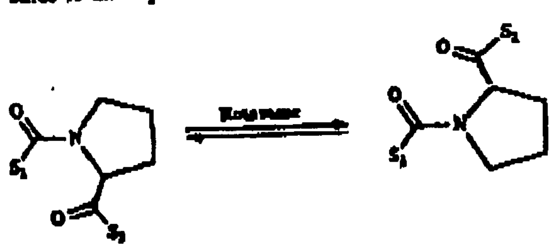

Fig 4. Cyclophilin and macrophilin FKBP are peptidyl-prolyl-cis-trans-isomerases (rotamases).

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INHIBITION OF IN VITRO IMMUNOGLOBULIN PRODUCTION BY RAPAMYCIN[1]

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Like FK506, rapamycin, a structural analog of FK506, is a strong immunosuppressant. The immunosuppressive effect of Rapa in in vitro IgG, IgM, and IgA production by human lymphocytes was examined in this study. To inhibit spontaneous or pokeweed mitogen–stimulated production of Ig by human peripheral blood lymphocytes, about one thousandfold lower concentrations of Rapa (IC_{50} = 0.3 nM–2 nM) were required than of cyclosporine (IC_{50} = 0.3 μM–2 μM). T cells were the direct targets of Rapa, because preincubation of T cells with Rapa abolished the T cells helper effect to T-dependent Ig production. Rapa also had direct suppressive effect on B cells, since Rapa suppressed IgG production by pure B cells stimulated with IL2 and *Staphylococcus aureus* Cowan I. Kinetic studies measuring IgG production and cell proliferation revealed that Rapa acted at the activation stage of T and B cells. Exogenous IL2 substantially reversed the inhibitory effect of CsA but not that of Rapa in Ig production. This study is the first report on the strong suppressive effect of Rapa on human humoral immune response with a quantitative comparison with that of CsA. The underlying mechanisms are also explored. The results indicate the potential usefulness of this drug in treatment of presensitized transplantation patients, with whom cytotoxic Ab is a major obstacle to a successful transplantation.

Rapamycin is a lipophilic natural macrolide produced by *Streptomyces hygroscopicus* NRRL 5491 (*1*). It was first reported in 1975 as an antifungal, antibiotic, and antitumor drug (*2, 3*). Subsequently, its immunosuppressive effect was documented by Martel (*4*), who showed that Rapa inhibited experimental allergic encephalitis and adjuvant arthritis in rats.

Rapa is similar in structure to FK506 (*5*), a new immunosuppressant that is tenfold to one hundredfold more potent than cyclosporine. Several groups (*6, 7*) have now shown separately that Rapa is fiftyfold to eightyfold more effective than CsA in rat heart and kidney transplantation. Similar results have also been obtained in large animals such as pigs and dogs (*6*). Rapa is also able to induce graft tolerance (*6, 7*) and is effective in reversing ongoing rejection of kidney, pancreas, and heart allografts in rats (*8*).

Rapa strongly suppresses T cell proliferation in mitogen- or anti-CD3/PMA–stimulated cultures, with one hundredfold the efficacy of CsA (*9*). However, unlike CsA or FK506, Rapa does not suppress early-phase T cell activation genes such as IL2, IL3, IL4, TNFα, IFN-γ, GM-CSF, c-myc, or c-fos (*10*). To date there is no documentation of the effect of Rapa on human immunoglobulin production.

In this study, we demonstrate that Rapa strongly suppresses in vitro immunoglobulin production by human peripheral blood mononuclear cells, and that both T cells and B cells are its direct targets. We also show that the suppressive effect of CsA, but not of Rapa, in Ig production can be partially reversed by IL2.

MATERIALS AND METHODS

Reagents. Human recombinant IL2 was purchased from Genzyme Co. (Boston, MA), pokeweed mitogen from Gibco/BRL (Gaithersburg, MD), *Staphylococcus aureus* Cowan I (SAC)* from Calbiochem (La Jolla, CA), and Lymphoprep from NYCOMED (Oslo, Norway). RPMI 1640, fetal calf serum, penicillin-streptomycin, and L-glutamine were ordered from Flow Laboratory (McLean, VA). RAPA was a gift from Wyeth-Ayerst Research (Princeton, NJ), and CsA a gift from Sandoz, Canada (Laval, Quebec).

Cell culture. PBMC were isolated from the heparinized blood of healthy donors with Lymphoprep gradient according to the manufacturer's instructions. T cells were obtained by rosetting PBMC with 2-aminoethylisothiouronium bromide (2-AET)–treated sheep red blood cells and subsequently by separating twice the rosette-forming cells on Lymphoprep gradient (*11, 12*). This T cell fraction normally contained < 1% B cells ($CD20^+$) and < 1% monocytes ($MO2^+$). The nonrosetting cells were further depleted of T cells with one more cycle of SRBC rosetting, and the final fraction represented non-T cells that normally contained < 1% $CD2^+$ cells, 30–40% $CD20^+$ cells, and 40–50% $MO2^+$ cells. For preparation of tonsillar B cells (*12*), mononuclear cells were first isolated by centrifugation of gently dispersed tonsillar cells over Lymphoprep. T cells were depleted with two cycles of SRBC rosetting. This B cell-enriched population contained 98–99% B cells ($CD20^+$), < 1% T cells ($CD2^+$), and < 1% monocytes ($MO2^+$). For in vitro Ig production, cells were cultured in 48-well plates in RPMI 1640 supplemented with 10% FCS, 2 mM L-glutamine, and antibiotics. Samples were in duplicate. All the experiments were repeated 3 times or more, although only the results of representative ones are shown.

Radioimmunoassays. IgG, IgM, and IgA in the culture supernatants were measured with RIA as described in a previous publication (*13*). Briefly, 96-well polyvinyl microtiter plates (Dynatech, Alexandria, VA)

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* Abbreviations: CHX, cycloheximide; SAC, *Staphylococcus aureus* Cowan I.

were coated with 100 μl of IgG fraction of sheep antisera specific to γ, μ, or α chains in carbonate buffer (pH 9.0). After the plates were blocked with Hanks' solution containing 10% FCS, 75 μl of test samples was added to the wells. After overnight incubation, the wells were washed and reacted with 75 μl of radiolabeled affinity-purified sheep antibodies to human γ, μ, or α chains. The plates were finally washed, and wells were cut and counted individually in a γ counter.

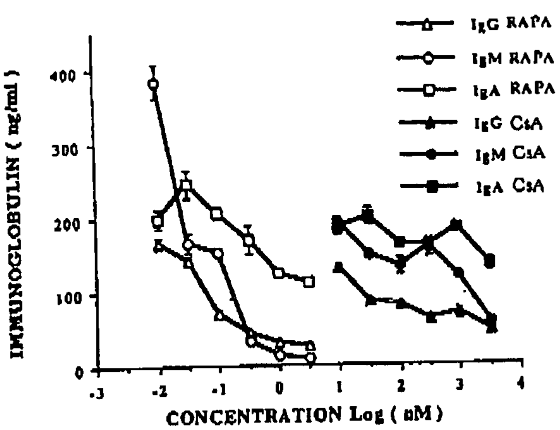
Assays for ^{3}H-thymidine uptake. ^{3}H-thymidine uptake was used as an indicator for cell proliferation. T cells from PBMC or B cells from tonsils were cultured at 2×10^5 cells/200 μl/well in 96-well plates. Cells were pulsed with ^{3}H-thymidine (0.5 μCi/well) for 6 hr before harvest and thymidine uptake was measured in a β scintillation counter. Samples were in quadruplicate.

RESULTS

Strong inhibition by Rapa of spontaneous and PWM-stimulated Ig production by PBMC of normal individuals. The effect of Rapa on Ig production was studied in in vitro spontaneous and PWM-stimulated cultures of PBMC. In a 7-day culture, Rapa at 1 nM suppressed spontaneous IgG, IgM, and IgA production by 81%, 90%, and 43%, respectively (Fig. 1A), and suppressed PWM-stimulated IgG, IgM, and IgA production by 37%, 48%, and 69%, respectively (Fig. 1B). In both cases, Rapa was about one thousandfold more potent than CsA to cause 50% inhibition of maximal production. To exclude the possibility that Rapa suppressed the PWM-stimulated Ig production by shifting the optimal responsiveness of PBMC to PWM, different concentrations of PWM were used to stimulate PBMC. Figure 2 shows that this is unlikely, since the optimal dose of PWM (0.016%) for IgG stimulation was not altered in the presence of 0.1 nM Rapa, and the inhibitory effect of Rapa cannot be reverted by any concentrations of PWM.

We next examined the kinetics of the inhibitory effect of Rapa on Ig production. Total PBMC from normal individuals were cultured for 12 days in the absence or presence of 0.25% PWM. Rapa (1 nM), CsA (1 μM), or cycloheximide (CHX, 10 μg/ml) was added to the cultures at day 0 (the day cultures started), 2, 4, 6, 8, or 10. Supernatants were collected at day 12 and measured for IgG. Figure 3A shows that spontaneous IgG production occurred predominantly between day 4 and day 10 according to the decreased inhibitory effect of CHX when it was added late in the culture. Addition of Rapa at day 8 or CsA at day 6 no longer had inhibitory effect on the IgG levels. In PWM-stimulated cultures (Fig. 3B), the major portion of the accumulated IgG was produced between day 4 and day 8 according to the cultures with CHX, and both Rapa and CsA were only effective in suppressing Ig production when added to the culture before day 2. These results suggest that Rapa acts on the early stage of T and/or B cell activation which leads to Ig production. The different kinetics of inhibition between spontaneous and PWM-stimulated PBMC probably reflect the different speeds of activation by mitogenic components in the culture medium (such as FCS) versus by PWM, since the spontaneous proliferation of PBMC peaks at day 5, while proliferation of the PWM-stimulated PBMC peaks earlier at day 3 (data not shown).

T cells as direct targets of Rapa. To determine the direct target cells of Rapa in the process of Ig production, we first studied the effect of Rapa on T cells. PBMC were separated into highly purified T cell (more than 98% CD2$^+$) and non-T cell (< 1% CD2$^+$) fractions and cultured separately. After 72 hr, T cells were extensively washed and returned to non-T cell fraction at a ratio of 2:1. The mixtures were cultured for an additional 7 days, and the IgG levels were measured. The results as shown in Figure 4 (columns 1 and 2) indicated that without T cells, the IgG production by non-T cells was not enhanced by PWM. This was consistent with the notion that PWM-stimulated Ig production is a T cell–dependent process (*14*), and it also indicated that the contamination of T cells in the non-T fraction was minimal. When the T cells preincubated in the medium were returned to non-T cells and the mixture was subsequently stimulated with PWM, the IgG production increased by more than twofold as expected (column 3), while T cells preincubated with 1 nM Rapa failed to respond to PWM and did not provide help to non-T cells (column 4). T cells activated by PWM for 72 hr followed by extensive wash could enhance IgG production by non-T cells in the subsequent culture (column 5), although the IgG level in this instance was



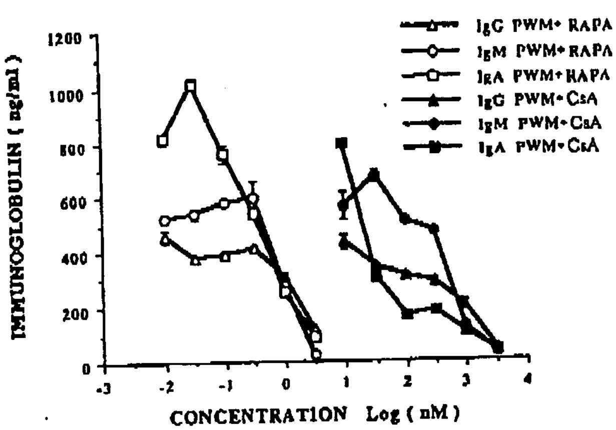


FIGURE 1. Rapa inhibits spontaneous and PWM-stimulated Ig production by PBMC. PBMC (0.75×10^6 cells/ml/well) were cultured either in medium (Fig. 1A) or in medium supplemented with 0.25% PWM (Fig. 1B). Various concentrations of Rapa or CsA as indicated were added at the beginning of the cultures. Supernatants were collected at day 7 and Ig levels were measured with RIA. The experiment was repeated more than three times, and results of a representative experiment are shown. The results are expressed as mean ± 1 SD.

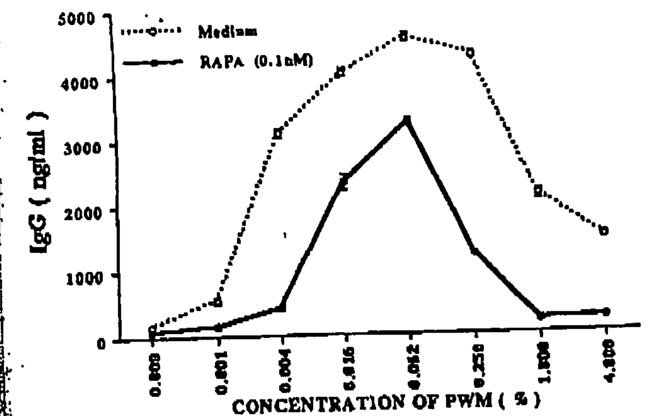


FIGURE 2. Rapa does not alter the optimal responsiveness of PBMC to PWM. PBMC (0.75×10⁶ cells/ml/well) were cultured for 7 days with various concentrations of PWM in the absence or presence of Rapa (0.10 nM). Supernatants were collected at the end of culture and measured for IgG with RIA. The experiment was repeated more than three times, and results of a representative experiment are shown. The results are expressed as mean ± 1 SD.

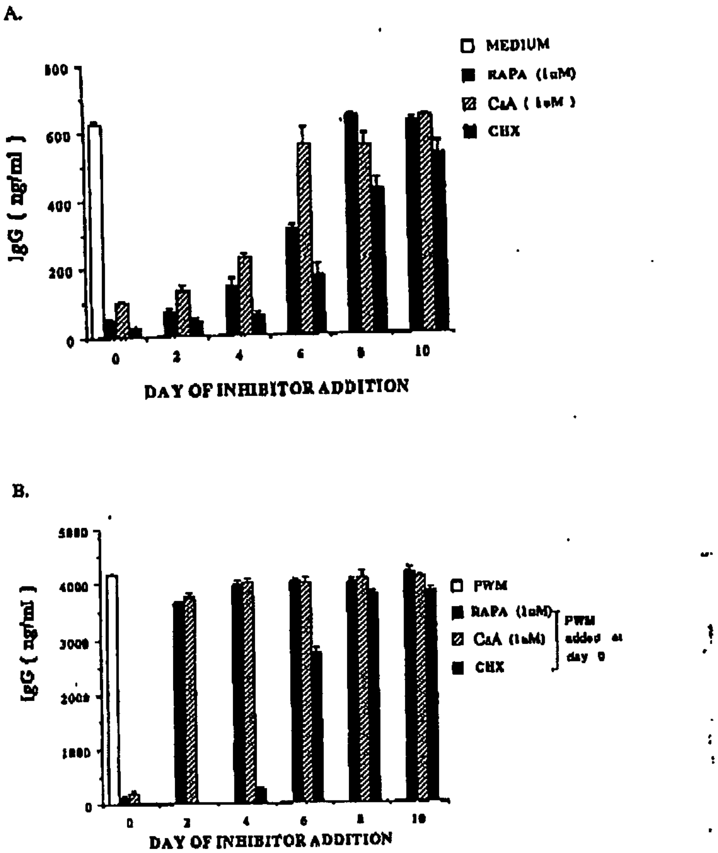


FIGURE 3. Time course of Rapa in inhibition of spontaneous or PWM-stimulated IgG production by PBMC. PBMC (0.75×10^6 cells/ml/well) were cultured for 12 days in the absence (Fig. 3A) or presence (Fig. 3B) of 0.25% PWM. Rapa (1 nM), CsA (1 μM), or cycloheximide (10 μg/ml) was added to the cultures at day 0 (the day cultures started), 2, 4, 6, 8, or 10. Supernatants were collected at day 12 and measured for IgG with RIA. The experiment was repeated more than three times, and results of a representative experiment are shown. The results are expressed as mean ± 1 SD.

lower than that in the sample where T cells were not treated but PWM was added in the second stage (column 5). This may be due to the fact that 3-day preincubation with PWM has already let T cells pass the optimal period to help B cells in terms of lymphokines or cell-cell interaction. When T cells were preincubated with PWM plus Rapa (1 nM), they were not able to help B cells at all (column 6). These results demonstrated that T cells were direct targets of Rapa in the process of T-cell-dependent Ig production, and that Rapa was able to condition resting T cells or mitogen-activated T cells to abolish their helper activity for Ig production.

Direct inhibitory effect of Rapa on B cell Ig production. We next investigated whether Rapa had a direct effect on B cells, by employing a T-cell-independent Ig production system. Highly purified tonsillar B cells (> 98% $CD20^+$, < 1% $CD2^+$) were stimulated with IL2 and SAC, and IgG and IgM were measured 7 days later. As shown in Figure 5, Rapa inhibited production of IgG and IgM in a dose-dependent fashion. At 1 nM, it inhibited IgG and IgM production by 91% and 95%, respectively. The kinetics of the inhibition was also studied (Fig. 6). Experiments with CHX again suggested that most of the IgG was produced between day 2 and day 6. Strong inhibitory effect, however, was only observed when Rapa was added at the beginning of the culture (Fig. 6). If added at day 2 or later, the inhibitory effect was greatly diminished. These results showed that B cells were also direct targets of Rapa, and that Rapa acted predominantly at the early stage of B cell activation triggered by IL2 plus SAC.

Susceptibility of B cells in the early activation stage to antiproliferative effect of Rapa. Resting B cells need to undergo the proliferation process to differentiate into Ig-producing cells (*15, 16*). We examined the effect of Rapa on B cell proliferation in order to understand the relationship between the antiproliferative effect of Rapa and its suppressive effect on Ig production. Rapa at 1 nM had moderate antiproliferative effect for IL2 and SAC-stimulated tonsillar B cells in the first 3 days of culture, and no longer had such an effect after day 4 (Fig. 7A), although the IgG production in a 5-day culture was still suppressed dose-dependently (Fig. 7B). To exclude the possibility that Rapa's failure of suppression of B cell proliferation measured at or later than day 4 was due to its loss of biological activity during the culture period, fresh Rapa was added to the culture at different dates. The results (Fig. 7C) showed that, if Rapa was freshly added at day 2 or day 3 and cells harvested at day 4 or day 5, respectively, it failed to suppress thymidine uptake, although its suppressive effect was obvious when Rapa was added at day 0 and cells were harvested at day 2. These results demonstrated that B cells in the early activation stage are susceptible to Rapa's antiproliferative effect.

Partial reversal by IL2 of suppressive effect of CsA, but not of Rapa, on Ig production. We next examined whether inhibition of lymphokine production was one of the mechanisms for Rapa's effect. IL2 was added to PBMC in the presence of CsA or Rapa, and IgG was measured 7 days later (Fig. 8). IL2 was able to partially reverse the suppressive effect of 1 μM CsA, but it had no rescue effect on the cultures suppressed by 1 nM Rapa. Additional experiments indicated that IL2 was not able to reverse the suppression by any lower dose of Rapa (data not

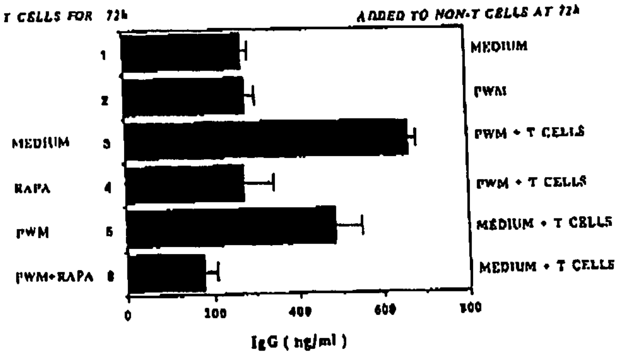


FIGURE 4. Pretreatment of T cells with Rapa abolishes their helper activity for Ig production. T cells from PBMC were preincubated for 72 hr with medium, Rapa (1 nM), PWM (0.25%), or Rapa plus PWM as indicated on the left. After extensive wash, T cells were returned to non-T fraction at a ratio of 2:1 (T:non-T). PWM (0.25%) were added to some cultures as indicated on the right. The mixture (0.75×10^6 cells/ml/well) was cultured for an additional 7 days and IgG levels in the supernatants were measured with RIA. For columns 1 and 2, no T cells were added for the secondary culture. The experiment was repeated more than three times, and results of a representative experiment are shown. The results are expressed as mean ± 1 SD.

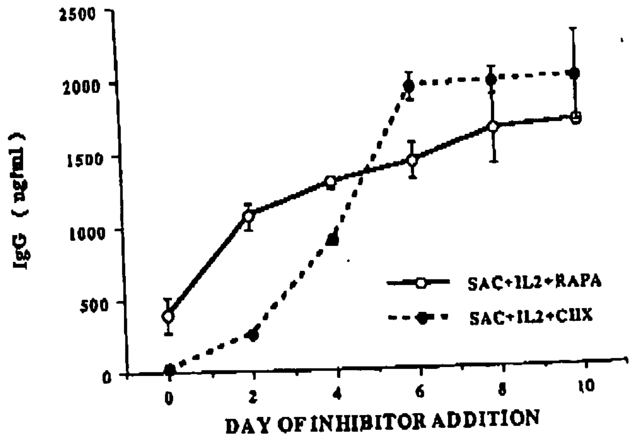


FIGURE 6. Time course of Rapa in inhibition of Ig production by IL2 and SAC-stimulated tonsillar B cells. Tonsillar B cells (0.5×10^6 cells/ml/well) were cultured for 12 days in the absence or presence of SAC (1/60,000) and IL2 (50 U/ml). Rapa (1 nM) or cycloheximide (10 μg/ml) was added to the cultures at day 0, 2, 4, 6, 8, or 10. Supernatants were collected at day 12 and measured for IgG. The IgG levels in unstimulated or SAC plus IL2-stimulated cultures were 1001±47 ng/ml and 1813±279 ng/ml, respectively. The experiment was repeated more than three times, and results of a representative experiment are shown. The results are expressed as mean ± 1 SD.

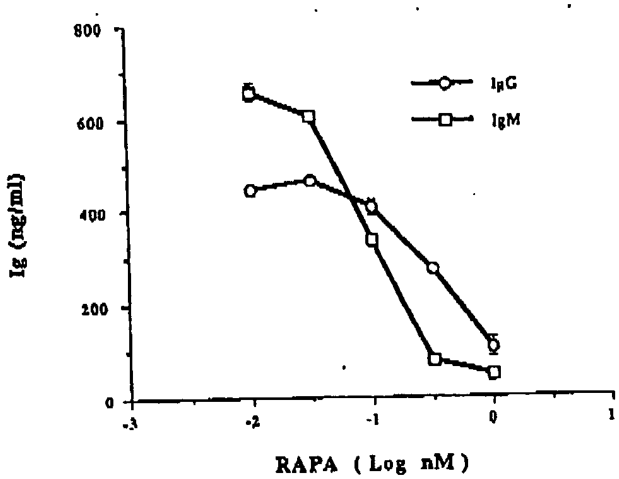


FIGURE 5. Rapa inhibits IL2 plus SAC-stimulated Ig production by tonsillar B cells. Tonsillar B cells (0.5×10^6 cells/ml/well) were cultured with 50 U/ml IL2 and 1/60,000 SAC in the presence of Rapa of different concentrations. Supernatants were collected at day 7 and measured for IgG and IgM with RIA. The experiment was repeated more than three times, and results of a representative experiment are shown. The results are expressed as mean ± 1 SD. The spontaneous IgG and IgM production by B cells was 267.8±34.2 and 89.1±11.2 ng/ml, respectively.

shown). Moreover, none of IL4, IL6, nor IFN-γ was able to reverse Rapa's inhibitory effect on Ig production (Table 1).

DISCUSSION

The present study for the first time reports the inhibitory effect of Rapa on human Ig production, and provides a quantitative comparison of the efficacy between Rapa and CsA. Martel et al. (*4*) in 1977 documented that Rapa suppresses passive cutaneous anaphylaxis in rats, although the nature of the Ab was not characterized. Recently, Wicker et al. (*17*) showed that Rapa inhibits murine B cell activation, but its effect on B cell function in terms of Ig production was not studied.

Our data show that Rapa-pretreated T cells are attenuated in their capability of providing help to non-T cells for Ig production in the secondary culture (Fig. 4). Rapa is a hydrophobic molecule. It can probably enter cells within 5 min and antagonize the effect of FK506 on histamine release from mast cells (*18*). However, a short period (30 min) of preincubation of T cells with the drug had no obvious effect on T cells helper activity for non-T cells (data not shown), but the effect became significant when preincubation was prolonged to 48 hr or 72 hr (Fig. 4). Such observations suggest that the effect of Rapa in this system is directly on T cells but is not due to releasing of intracellular or membrane-bound Rapa from pretreated T cell to non-T cells during the second stage of culture. This experiment (Fig. 4) further indicates that Rapa has an effect on unstimulated T cells (Fig. 4), and the molecule(s) or pathways mediating Rapa's action seem to exist in both resting and activated T cells. Our data also demonstrated that Rapa can directly inhibit B cell function in terms of Ig production in a model where B cells were stimulated by IL2 and SAC in the absence of T cells. Contrarily, CsA at one thousandfold higher concentration failed to consistently suppress Ig production in this model (data not shown).

The mechanism of Rapa's effect is still under investigation. The drug seems to act at the activation stage of T and B cells. Rapa has been shown to inhibit Con A–stimulated proliferation of murine T cells only when added within the first 24 hr (*9*). Similarly, Rapa significantly suppresses anti-IgM–stimulated proliferation of murine B cells only when added within the first 40 hr (*17*). In those systems, T or B cells normally do not enter their peak of thymidine uptake until 48 hr (*17, 19*). Our results have substantiated such observations in the human model. Indeed, Rapa suppresses PWM-stimulated Ig production,

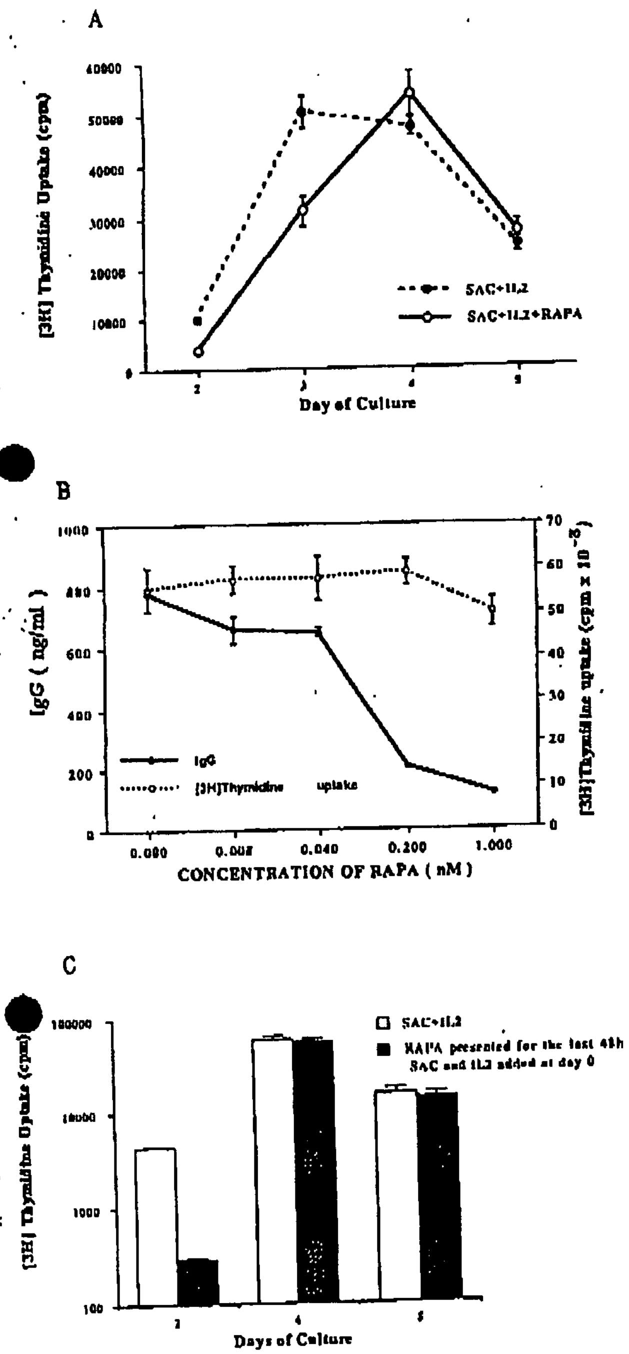


FIGURE 7. Antiproliferative effect of Rapa on SAC plus IL2–stimulated tonsillar B cells. (A) Time course of the antiproliferative effect of Rapa. SAC (1:60,000), IL2 (50 U/ml), and Rapa (1 nM) were added to the culture at day 0 and cells were harvested at day 2, 3, 4, or 5. Cells were pulsed with ^{3}H-thymidine for the last 6 hr of the culture period. (B) Effect of Rapa on IgG production and proliferation of SAC (1:60,000) plus IL2 (50 U/ml)–stimulated tonsillar B cells in a 5-day culture. All the reagents were added at day 0. Supernatants were harvested at day 5 for IgG measurement, and cells were also harvested at day 5 with a 6-hr pulse of ^{3}H-thymidine. (C) SAC (1:60,000) and IL2 (50 U/ml) were added at the beginning of the culture. Rapa (1 nM) was added at day 0, day 2, and day 3 and the thymidine uptake was measured at days 2, 4, and 5, as indicated. Cells were pulsed with ^{3}H-thymidine for the last 6 hr of the culture.

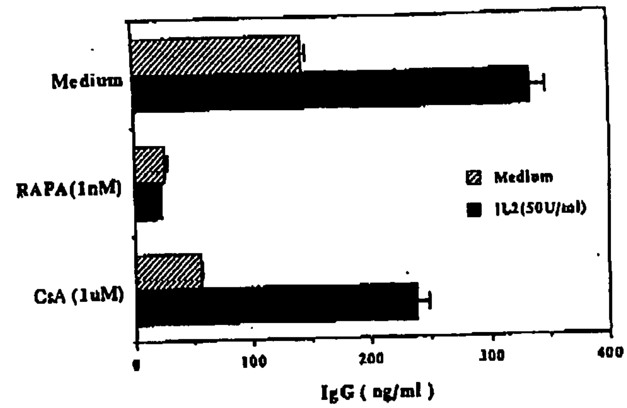


FIGURE 8. The suppressive effect of CsA, but not of Rapa, on Ig production can be partially reversed by IL2. PBMC (1×10^6 cells/ml/well) were cultured in the presence of Rapa (1 nM) or CsA (1 μM) as indicated on the left. IL2 (50 U/ml) was added at the beginning of the culture. Supernatants were collected at day 7 and measured for IgG with RIA. The experiment was repeated more than three times, and results from a representative experiment are shown. The results are expressed as mean ± 1 SD.

TABLE 1. Nonreversal by IL4, IL6, and IFN-γ of effect of Rapa on IgG production[a]

	Added to the cultures	IgG (ng/ml)
Experiment 1	—	143.2±3.7
	Rapa	25.2±3.4
	Rapa + IL6	23.7±3.2
	Rapa + IFN-γ	21.6±2.6
Experiment 2	—	67.1±6.8
	Rapa	8.5±0.5
	Rapa + IL4	10.1±0.9
Experiment 3	—	273.6±8.5
	PWM	1344.0±25.3
	PWM + Rapa	26.0±1.0
	PWM + Rapa + IL6	35.3±0.3
	PWM + Rapa + IFN-γ	27.9±1.7

[a] PBMC (0.75×10^6 cells/ml/well) were cultured for 7 days. Rapa (1 nM), PWM (0.25%), IL4 (15 ng/ml), IL6 (100 U/ml), and IFN-γ (1000 U/ml) were included as indicated. Supernatants were collected at the end of culture and measured for IgG with RIA.

which is a T-cell–dependent process, only if it is added before day 2 of the cultures. Proliferation and Ig production by SAC plus IL2–stimulated pure tonsillar B cells are also only susceptible to the inhibitory effect of Rapa within the first 2 days of culture. These results imply that Rapa's effect is cell stage–specific and is not generally cytostatic or cytotoxic. Furthermore, these data suggest that in vivo Rapa may be more effective when administered early in T-cell–mediated immune response and may have less impact against fully activated T and B cells in terms of antibody formation.

One special feature of Rapa is that, unlike CsA and FK506, the drug has little inhibitory effect on the IL2, IL4, and IFN-γ production by mitogen-activated T cells (*10, 20*). B cell acti-

vation and differentiation is a multistage event regulated by several lymphokines. IL2 acts as a competent growth and differentiation factor for human B cells (*21, 22*), and part of its effect is mediated by the B cell autocrine IL6 (*21*). IL4 plays a role as a cocompetence factor for anti-μ activation of B cells (*23*). IFN-γ primes B cells in a manner that amplifies subsequent mitogenesis (*24*), and it also regulates the isotype switch in the B cell differentiation (*25*). IL6 is one of the essential late-acting factors in PWM-induced Ig production (*26*). We employed lymphokine supplementation experiments to examine whether suppression of the production of these lymphokines is one of the mechanisms for Rapa's effect in Ig synthesis. Our data indicate that none of IL4, IFN-γ, nor IL6 is able to reverse the inhibitory effect of Rapa in unstimulated or PWM-stimulated PBMC. Therefore, the possibility that Rapa suppresses Ig production via suppressing the production of these lymphokines is unlikely. IL2 partially reversed CsA-induced inhibition of spontaneous Ig production by PBMC, but it was not able to rescue the inhibitory effect. This observation confirms one of the major mechanisms of action of CsA and highlights the notion that Rapa does not exert its immunosuppressive effects via a reduction of IL2 synthesis.

The strong suppression of Ig production may contribute to the effect of Rapa in preventing allograft rejection. Currently, we are examining the effect of Rapa on Ig production in an in vivo rat model, with special interest in the secondary humoral response, which is more relevant in presensitized patients.

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THE EFFECTS OF IMMUNOSUPPRESSIVE AGENTS ON IN VITRO PRODUCTION OF HUMAN IMMUNOGLOBULINS[1]

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We have evaluated the effects of CsA, methylprednisolone (MP), 6-mercaptopurine (6-MP), and FK506 on T cell–dependent and T cell–independent immunoglobulin production. FK506 and 6-MP were potent inhibitors of IgG and IgM production by PWM-stimulated peripheral blood mononuclear cells, which depend on the presence of T cells. CsA was less effective in this system and MP actually enhanced IgG and IgM production. In order to assess the direct effects of these various immunosuppressive agents on B cells, we utilized human B cell lines representing different stages of B cell differentiation. The B cell lines CESS and SKW6.4 exhibit increased production of IgG and IgM, respectively, in response to interleukin-6. These cells represent activated, but not fully differentiated, B cells. CsA inhibited IL-6-induced IgG production by CESS cells by 64% at 100 ng/ml and 6-MP inhibited this response by 82% at 250 ng/ml. Neither CsA nor 6-MP effectively inhibited IL-6-induced IgM production by SKW6.4 cells. MP at 250 ng/ml inhibited IL-6-induced IgG production by 89%, but enhanced IL-6-induced IgM production more than twofold. FK506 did not inhibit IL-6-induced IgG or IgM production, suggesting that it has no direct effect on the ability of B cells to respond to this differentiation factor. These experiments clearly demonstrate that CsA, MP, and 6-MP have direct inhibitory effects on the response of human B cells to IL-6. In contrast, FK506 has no direct effect on these B cells lines, but is more potent than the other agents at inhibiting T cell–dependent immunoglobulin production.

There has been marked improvement in the survival of organ allografts since the introduction of CsA into clinical therapeutic protocols. The improvement is probably due to the potent inhibitory effects of CsA on T cell responses, which are thought to be the predominant cause of acute rejection in the early posttransplant period. Although the involvement of humoral immunity in graft rejection has also been documented (1–3), the effects of CsA and the other immunosuppressive agents on B cell responses are poorly understood. CsA, FK506, and the purines have been shown to inhibit immunoglobulin production in vitro (*4–7*), while steroids have been reported to enhance this response (*8, 9*). The difficulty in assessing the effects of these agents on B cell responses is due to the fact that B cell activation, growth, and differentiation depend on the presence of interleukins 4, 5, and 6, which are produced by T cells and macrophages (*10–12*). For example, the production of immunoglobulins by PWM-stimulated B cells has been shown to be dependent on T cell help (*13–16*). Thus, it is often difficult to distinguish between the direct effects of an immunosuppressive agent on B cells and indirect effects mediated through action on accessory cells. The present study was performed to com

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the effects of CsA, FK506, 6-mercaptopurine (6-MP),* and methylprednisolone (MP) on the production of immunoglobulins by normal human B cells and B cell lines representing different stages of maturation. The CESS and SKW6.4 cell lines respond to interleukin-6 (IL-6) by up-regulating immunoglobulin production and represent activated, but not fully differentiated, B cells. IM-9 and RPMI1788 are B cell lines that spontaneously secrete immunoglobulin and represent a stage in B cell maturation analogous to that of a plasma cell.

MATERIALS AND METHODS

Reagents. Interleukin 6 was purchased from Genzyme (Boston, MA). Purified CsA, a generous gift of Sandoz (E. Hanover, NJ), was reconstituted in absolute ethanol and further diluted in phosphate-buffered saline to working concentrations. Methylprednisolone sodium succinate (Upjohn Co., Kalamazoo, MI) was reconstituted in the diluent provided and further diluted in PBS. The 6-mercaptopurine (Sigma, St. Louis, MO) was reconstituted in warm (37°C) absolute ethanol and further diluted in PBS. FK506, a generous gift of Fujisawa Pharmaceuticals (Osaka, Japan), was reconstituted in absolute ethanol and further diluted in PBS to working concentrations.

Cells. CESS, SKW 6.4, RPMI1788, and IM-9 cell lines were obtained from the American Tissue Culture Collection (Rockville, MD). CESS is a human lymphoblastic cell line isolated from the peripheral blood of a patient with myelocytic leukemia (*17*) and responds to B cell differentiation factors by an increase in the number of IgG-secreting cells (*18*). SKW6.4 is an Epstein-Barr virus–positive human B cell line that responds to differentiation factors by an increase in the number of IgM-secreting cells (*19*). The RPMI1788 cell line originated by EBV infection of peripheral blood leukocytes of an apparently normal male (*20*) and spontaneously secretes IgM. The IM-9 cell line was initiated from a bone marrow sample taken from a female patient with multiple myeloma and spontaneously secretes IgG (*21*). All cell lines were maintained in RPMI 1640 (Whittaker M.A. Bioproducts, Walkersville, MD) supplemented with 50 U/ml penicillin, 50 μg/ml streptomycin, 25 μg/ml gentamicin, and 10% fetal bovine serum (FBS, Hyclone, Logan, UT).

Cell proliferation. Human peripheral blood mononuclear cells were isolated from the blood of normal volunteers by density-gradient centrifugation as previously described (*22*). DNA synthesis was measured by adding 0.6 μCi ^{3}H-TdR (EI DuPont, North Billerica, MA; specific activity 2 Ci/mmol) per 200 μl of cells for 18 hr, and then harvesting onto glass fiber filters. The filters were dried and then suspended in 2 ml of scintillation fluid, and counted in a scintillation counter (Beckman LS1801).

Immunoglobulin production by B cell lines. IM-9, RPMI1788, CESS, and SKW 6.4 were cultured in 24-well plates at a concentration of 5×10^5 cells/ml. Supernatants from IM-9 and RPMI 1788 cells were collected after 48 hr and assayed for IgG and IgM, respectively. The mean IgG production by IM-9 was 268.6±214 ng/10^6 cells and the mean IgM production by the RPMI 1788 cell line was 143.5±39.6 ng/10^6 cells. IL-6 did not increase the level of immunoglobulin produced by these cell lines. Recombinant IL-6 (50 U/ml) was added to CESS or SKW 6.4 cells at the initiation of culture, and supernatants were collected after 48 hr to assay for IgG and IgM, respectively. The mean IgG production by the CESS cell line after stimulation with IL-6 was 156.9±98.6 ng/10^6 cells, representing a 28% increase over unstimulated cultures ($P<0.005$). Mean IgM production in the SKW 6.4 cell line was 68.1±23.9 ng/10^6 cells, representing a 187% increase over unstimulated IgM levels ($P<0.0004$). Background IgG and IgM levels were subtracted, with the results representing the increase in immunoglobulin production upon stimulation with IL-6.

Pokeweed mitogen–induced IgG and IgM production by PBMC. PBMC were cultured at 10^6 cells/ml in RPMI 1640 supplemented with 10% FBS in 24-well plates and stimulated with 100 μg/ml PWM (Gibco, Grand Island, NY). Supernatants were collected on day 7 and assayed for IgM and IgG. Mean IgM production was 93.9±45.4 ng/10^6 cells and IgG production was 292.0±282 ng/10^6 cells. Background levels of IgM and IgG production in unstimulated cultures were subtracted.

ELISA measurement of human IgM and IgG in cell culture supernatants. Enzyme-linked immunosorbent assays were used to quantitate IgM and IgG levels in supernatants from PBMC and B cell lines. MicroELISA plates (Immulon I, Dynatech) were coated with 100 μg (0.1 ml) of goat antihuman IgG or IgM (Tago, Burlingame, CA) in 40 mM Na_2CO_3, 90 mM $NaHCO_3$, 8 mM NaN_3 overnight at 4°C. On the following day, the plates were washed with 0.025% Tween 20 with 0.02% NaN_3 using a Skatron miniwash. The cell supernatants were added to wells and incubated at room temperature for 2 hr. The plates were then washed extensively, and 100 μl of goat antihuman IgM or IgG conjugated to alkaline phosphatase (Tago), diluted as suggested by the manufacturer in 0.13 mM EDTA, 3 mM NaN_3, 0.05% Tween 20, and 0.25% BSA fraction V, was added to each well. The plates were then incubated for 2 hr at room temperature, washed, and 100 μg (0.1 ml) of p-nitrophenyl phosphate (Sigma) in 9.7% diethanolamine, 3 mM NaN_3, and 0.5 mM $MgCl_2$ was added. The plates were read by a microELISA plate reader (Dynatech) after 30 min. Quantitation of IgM and IgG was done by probit analysis using a standard curve of calibrated human serum (Sigma).

RESULTS

Effects of CsA, FK506, MP, and 6-MP on PWM-stimulated IgM and IgG production by normal human PBMC. To assess the immunosuppressive potential of these agents on T cell–dependent B cell responses, the agents were added to PBMC cultures 1 hr prior to the addition of PWM. Figure 1 shows representative dose-response curves of the effects of these agents on PWM-stimulated IgM and IgG production. CsA, FK506, and 6-MP inhibited PWM-induced IgM and IgG production in a dose-dependent manner, but MP enhanced both IgG and IgM production. Adding CsA or FK506 24–48 hr after PWM progressively diminished the inhibitory effects (Fig. 2), suggesting that they inhibit early events in B cell activation or that they affect T cell or monocyte responses. In contrast, 6-MP was inhibitory regardless of when it was added to the culture, indicating that its action is not necessarily dependent

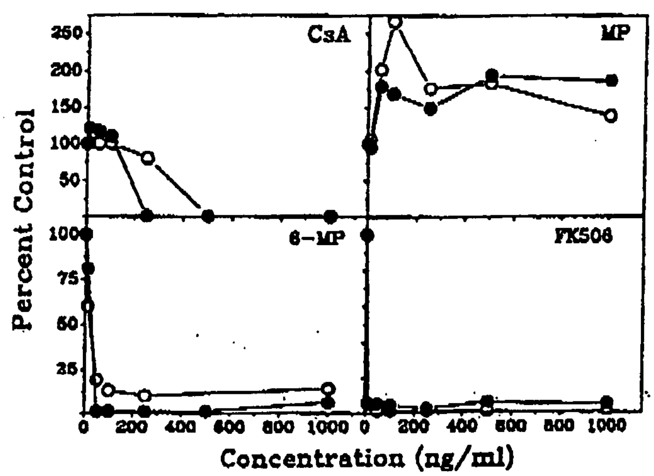


FIGURE 1. Effects of CsA, steroids, 6-MP, and FK506 on PWM-induced immunoglobulin production by PBMC. Agents were added 1 hr prior to the addition of 100 μg/ml PWM, and supernatants were collected on day 7 to assay for IgM (○) and IgG (●) production by ELISA. Background IgM and IgG production by unstimulated cultures was subtracted. Data are representative of 4 separate experiments.

* Abbreviations: MP, methylprednisolone; 6-MP, 6-mercaptopurine.

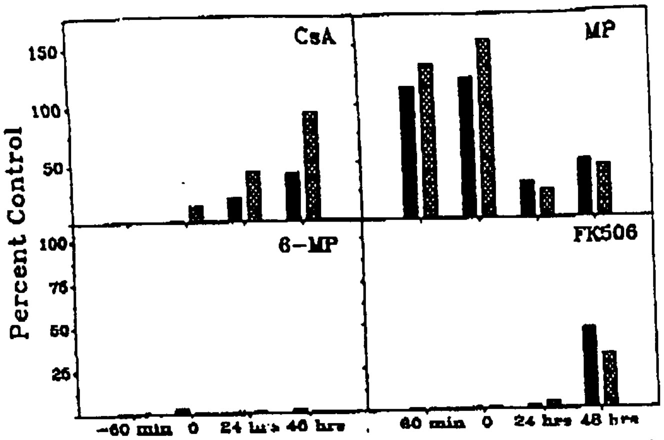


FIGURE 2. Effects of time of addition of immunosuppressive agents on PWM-induced immunoglobulin production by PBMC. Agents at 1000 ng/ml were added at the times indicated relative to the addition of PWM. Supernatants were collected on day 7 to assay for IgM (filled box) and IgG (hatched box) production by ELISA. Data are representative of 3 separate experiments.

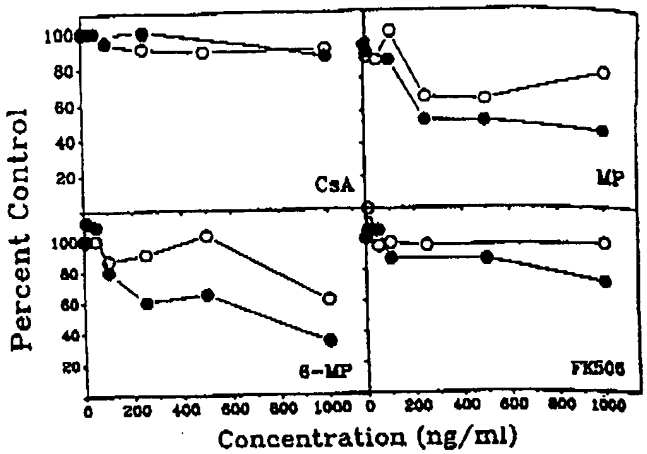


FIGURE 3. Effects of CsA, steroids, 6-MP, and FK506 on spontaneous immunoglobulin production by B cell lines. RPMI 1788 and IM-9 cell lines were cultured at 5×10[5] cells/ml for 48 hr with or without the agents indicated, at which time supernatants were collected from RPMI 1788 to assay for IgM (O) and from IM-9 to assay for IgG (●) production by ELISA. Data are representative of 3 separate experiments.

on the stage of B cell activation. Although MP enhanced immunoglobulin production when added prior to PWM, it inhibited this response when added 24–48 hr after the mitogen. This observation indicates a relatively complex regulation of immunoglobulin production by glucocorticoids.

Effects of CsA, FK506, MP, and 6-MP on spontaneous and IL-6-induced immunoglobulin production by B cell lines. To determine if the immunosuppressive agents had direct effects on B cells, we examined their effects on immunoglobulin production by B cell lines. As shown in Figure 3, CsA inhibited spontaneous IgG production by IM-9 cells and spontaneous IgM production by RPMI 1788 cells by <10%. FK506 was also relatively ineffective at inhibiting spontaneous IgG production, causing <20% inhibition at concentrations below 500 ng/ml. These data suggest that the CsA and FK506 would be relatively ineffective at inhibiting antibody production by plasma cells. MP and 6-MP inhibited spontaneous IgG production by 40–50% at 250 ng/ml, but IgM production was more resistant.

The addition of 50 units/ml IL-6 to CESS or SKW6.4 cells enhanced IgG and IgM production, respectively. CsA inhibited IL-6-induced IgG production by 64% at 100 ng/ml and 6-MP inhibited this response by 82% at 250 ng/ml (Fig. 4). This observation suggests that CsA has a direct effect on the response of B cells to IL-6. However, CsA and 6-MP had very little effect on IgM production induced by IL-6. MP inhibited IL-6-induced IgG production by 89% at 250 ng/ml, but enhanced IL-6-induced IgM production by greater than two-fold. This observation could be due to major differences in the regulation of IgM and IgG production or to differences in the two cell lines that produce these immunoglobulins. FK506 did not inhibit either IgG or IgM production in these cell lines, suggesting that its inhibitory effect on immunoglobulin production is not due to a direct effect on the ability of B cells to respond to IL-6.

All of the agents were tested for their effects on proliferation of the B cell lines. Agents were added at 1000 ng/ml at the initiation of culture and the effects on DNA synthesis determined. Only 6-MP inhibited the proliferation of these cell lines

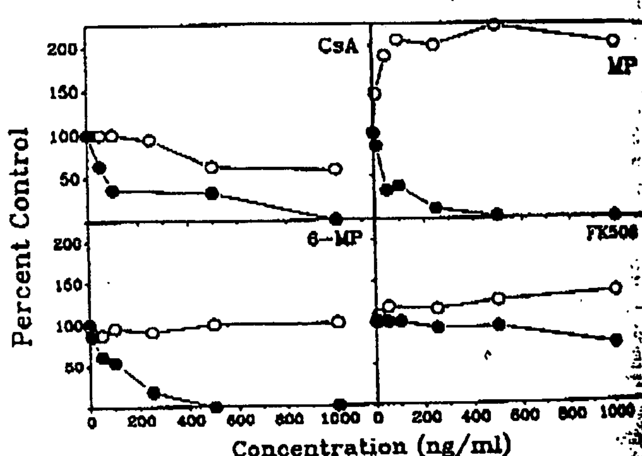
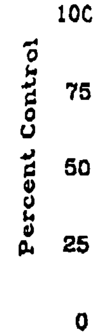


FIGURE 4. Effects of CsA, steroids, 6-MP, and FK506 on IL-6-induced immunoglobulin production by B cell lines. Agents were added 1 hr prior to the addition of 50 units/ml IL-6 to 5×10[5]/ml CESS or SKW6.4 cells. Supernatants were collected from SKW6.4 at 48 hr to assay for IgM (O) or from CESS to assay for IgG (●) production by ELISA. Background IgM and IgG production by unstimulated cultures was subtracted. Data are representative of 3 separate experiments.

(Fig. 5), which is consistent with the known effects of thiopurines on DNA, RNA, and protein synthesis.

DISCUSSION

The experiments reported here clearly demonstrate that CsA, 6-MP, and FK506 are potent inhibitors of T cell-dependent immunoglobulin production by B cells. However, using human cell lines representing more mature stages of B lymphocyte differentiation, we demonstrated that only CsA and 6-MP had direct inhibitory effects on the response of B cells to IL-6. In contrast, it has been reported that CsA has no effect on the response of activated human tonsillar B cells to a crude B cell differentiation factor preparation (23). These differences



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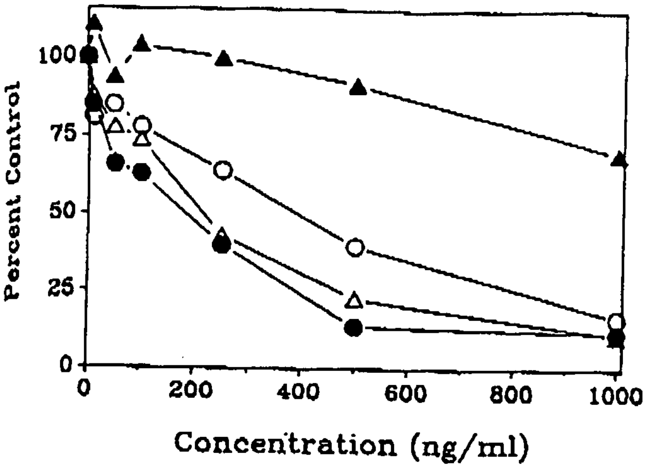

FIGURE 5. Effect of 6-MP on B cell line proliferation. Various concentrations of 6-MP were added to 5×10⁵ cells/ml of CESS (△), SKW6.4 (▲), IM-9 (○), and RPMI 1788 (●). Cells were pulsed with ³HTdR at 24 hr and harvested 18 hr later. Data are representative of 3 separate experiments.

be due to the fact that we quantitated immunoglobulin production rather than antibody-secreting cells. Although their data suggest that CsA did not decrease the number of cells responding, the amount of immunoglobulin produced by each cell may have been suppressed. Studies evaluating B cell responses in transplant recipients receiving CsA have shown that B cell proliferation and immunoglobulin production by PBMC stimulated with the B cell mitogen *Staphylococcus aureus* Cowan are significantly depressed in comparison with normal controls (*24*). The addition of IL-2 or B cell growth factor to these cultures enhanced the response but did not restore proliferation or immunoglobulin production to control levels. Our study suggests that these findings may be due to the ability of CsA to block the response of B cells to these growth factors. CsA and FK506 have been reported to inhibit the activity of different peptidyl-prolyl isomerases, both of which appear to be important in the proper folding of proteins that bind to the IL-2 gene enhancer region (*25–28*). However, the fact that FK506 did not inhibit IL-6-induced IgG production suggests a fundamental difference in some of their immunosuppressive effects.

The immunosuppressive effects of steroids were remarkably diverse. MP enhanced PWM-induced IgM and IgG production when added prior to the mitogen, but was inhibitory when added 24–48 hr later. This observation, coupled with the finding that MP inhibited spontaneous and IL-6-induced IgG production, suggests that glucocorticoids may inhibit antibody production primarily by a direct effect on B cells. However, MP enhanced IL-6-induced IgM production by SKW6.4 cells. This difference may reflect different regulatory pathways for IgM and IgG production. In support of this hypothesis, glucocorticoids have been shown to have both positive and negative effects on gene expression via interactions with steroid receptors and glucocorticoid response elements (*29, 30*). Alternatively, the different effects of steroids on IL-6-induced IgM and IgG production may be related to effects on IL-6-receptor expression in these two cell lines. Steroids have been shown to increase expression of IL-6 receptors on endothelial cell lines (*31*), but we have not yet studied this effect on the B cell lines.

The antimetabolite 6-MP inhibited PWM-induced IgM and IgG production regardless of when it was added to the culture. This finding indicates that the immunosuppressive effect of 6-MP is not limited to early activation events and may reflect inhibition of protein synthesis. The 6-MP inhibited spontaneous IgM and IgG production in B cell lines, and it also inhibited IL-6-induced IgG production by CESS cells, but had no effect on IL-6-induced IgM production by SKW6.4 cells. These results correlate with the effect of 6-MP on the proliferation of these cell lines, suggesting that the immunosuppressive effect of 6-MP may be associated with its antiproliferative properties.

Significant improvements in early graft survival have been achieved in transplantation in recent years. However, antibody-mediated rejection appears relatively resistant to current immunosuppressive protocols and may affect long-term graft survival (*32, 33*). Prevention or reversal of antibody-mediated rejection may therefore depend on the development of immunosuppressive protocols that affect late stages of B cell growth and differentiation.

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RADIATION-INDUCED AUGMENTATION OF HOST RESISTANCE TO HISTOCOMPATIBLE TUMOR IN MICE

DETECTION OF A GRAFT ANTITUMOR EFFECT OF SYNGENEIC BONE MARROW TRANSPLANTATION[1]

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Lethally irradiated and syngeneic bone marrow–reconstituted (C57BL/6J×DBA/2J) F_1 female mice demonstrated prolonged survival following challenge with the DBA/2 mastocytoma P815-X2 compared with nonirradiated littermate controls. This radiation-induced augmentation of host resistance to P815-X2 was not abolished by the adoptive transfer of normal syngeneic spleen cells. In addition, this phenomenon was not detectable in adult thymectomized recipients, suggesting the requirement for an intact host thymus. This effect was also absent in syngeneic F_1 male recipients. We suggest that lethal irradiation and marrow reconstitution may result in activation of a nonspecific immune effector mechanism against tumor cells—and, as such, may serve as a model to explore the graft-antitumor effect of bone marrow transplantation.

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The immunosuppressive effects of ionizing radiation are recognized in radiobiology and medicine. Recently, however, there has been growing interest in seemingly paradoxical immunostimulatory effects of radiation under certain circumstances (*1*). Stimulation of immune cell production by low-

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Experimental treatment of autoimmune MRL-lpr/lpr mice with immunosuppressive compound FK506

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SUMMARY

A newly developed immunosuppressive drug, FK506 (Fujisawa, Japan) is known to inhibit T-cell immunity. We have evaluated the action of this compound in MRL/lpr mice which develop a severe autoimmune disease. Eight-week-old female MRL/lpr of mice were treated subcutaneously with 2 mg/kg (high dose), 0·8 mg/kg (medium dose), 0·2 mg/kg (low dose) or solvent only (control) six times per week. Survival times of the mice were prolonged in the medium and the high dose treatment groups. The lymph node swelling was dramatically prevented with the high dose treatment. The increasing footpad swelling seemed to be also suppressed with the treatment. FACS analyses of the spleen cells revealed that FK506 reduced the percentage of double negative T cells (Thy-1.2$^+$, Lyt-2$^-$, L3T4$^-$). Serological studies showed that anti-ssDNA and anti-dsDNA activities were significantly reduced by the high dose treatment, which is different from recent findings with Cyclosporine A. The high dose treatment also suppressed the total amount of IgG, even though the IgG concentration was rather increased by the medium dose treatment. Decreased proteinuria as well as pathological evaluations of the kidneys and lungs indicated that there were marked ameliorations in these organs with the treatment. These results suggest that FK506 could be potentially used for the treatment of autoimmune diseases.

INTRODUCTION

MRL-lpr/lpr mice develop a spontaneous and aggressive autoimmune disease, which resembles human systemic lupus erythematosus (SLE). The characters of the generalized autoimmune disease include marked lymphadenopathy, auto-antibody production, lupus nephritis, interstitial pneumonitis, arthritis and premature death (Murphy & Roth, 1977; Andrew *et al.*, 1978). Neonatal thymectomy had a significant suppressive effect on the disorder (Steinberg *et al.*, 1980; Theofilopoulos *et al.*, 1981) and the lymphadenopathy consists primarily of the abnormal proliferation of T cells with a unique phenotype (Morse *et al.*, 1982). Thus, it is generally believed that T lymphocytes play an important role in the pathogenesis. However, other factors are involved in the development of this complex type of autoimmune disease (Davidson *et al.*, 1984).

Several immunosuppressive drugs, including Cyclosporine A, have been used to treat MRL-lpr/lpr mice (Smith, Chused & Steinberg, 1984; Isenberg *et al.*, 1981; Okudaira *et al.*, 1986; Mountz *et al.*, 1987). The results of these experiments have contributed to understanding the more precise mechanisms in the autoimmune phenomenon of the mouse. In the present study, we applied the newly developed immunosuppressive compound, FK506 (Kino *et al.*, 1987a, b; Thomson, 1989), which is known to inhibit especially T-cell immunity, to the experimental treatment of MRL-lpr/lpr mice. We found that FK506 had strong immunosuppressive effects on several aspects of the autoimmune disease.

MATERIALS AND METHODS

Mice

MRL-lpr/lpr mice (breeding pairs provided by Dr Murphy, the Jackson Laboratory, Bar Harbor, ME) were bred in the Central Institute for Experimental Animals (Kanagawa, Japan). The animals were specific pathogen free.

FK506 administration and the experimental protocols

FK506 (Fujisawa Pharm. Co. Ltd, Osaka, Japan) dissolved in carrier solvent (HCO-60 and D-mannitol) was diluted in phosphate-buffered saline (PBS). Eight-week-old female MRL-lpr/lpr mice were administered subcutaneously six times a week. For control, mice were given the equivalent dose of carrier solvent and PBS. For dose–response analyses, 2 mg/kg/day as a high dose, 0·8 mg/kg/day as a medium dose, and 0·2 mg/kg/day as a low dose treatments were used. Other experiments were

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...d out with the high dose treatment and the placebo ...ion. The adenopathy was evaluated as scores. The dia... of each palpable lymph node was measured and graded as ...s: 0, 0–5 mm; 1, 6–10 mm; 2, 11–15 mm; 3, 16–20 mm; ...25 mm. The total score of each mouse was the summation ... individual scores of the lymph nodes. Proteinuria was ...ined semi-quantitatively by colorimetric reaction with ...istix paper (Sankyo. Tokyo, Japan). The measurement of ...ind footpads was performed with a dial thickness gauge ...MFG. Tokyo, Japan).

...ogical evaluation
...ys and lungs were removed, fixed in 10% formalin and ...ssed according to standard techniques. These sections ...ained with hematoxylin and eosin.

...ogical analyses
... were assayed for anti-single stranded (ss) DNA or anti-...le stranded (ds) DNA activities of IgG and IgM classes by ...e-linked immunosorbent assay (ELISA) as described ...daira *et al.*, 1981). In brief, the denatured ssDNA was ...red by boiling a solution of calf thymus DNA (10 μg/ml, ...; Sigma, St Louis, MO). The dsDNA was produced by S1 ...ase treatment (Takara Shuzo, Kyoto, Japan). The DNA ... bound to the wells of microtitre plates which were ...eated with poly-L-lysin (Sigma). The plates were blocked ...1% bovine serum albumin (Sigma) plus 0·5% bovine ...ma globulin and serial diluted test sera were incubated. ...r washing peroxidase-conjugated goat anti-mouse IgM, or ...-mouse IgG antibodies (Cappel, U.S.A.) were added. The ...vity was measured by *O*-phenylene diamine solution (0·4 ...ml) and Titertex Multiskan. A mixed sera from old MRL-...lpr mice was used for a standard and 1000 units were the ...ivalent of each activity in this serum. The measurement of ...l immunoglobulin concentrations was performed by radial ...unodiffusion plates (The Binding Site, Birmingham, U.K.)

... staining and flow cytometry
...le cell suspensions of splenocytes were incubated with ...rescinated monoclonal anti-Thy-1.2, anti-Lyt-2, anti-L3T4 ...kon-Dickinson, U.S.A.) or anti-B220 (kindly supplied by ...K. Okumura, Juntendo University) antibodies and were ...lysed by a fluorescence-activated cell sorter (FACS).

...istical analysis
...istical significance of differences was analysed by Student's ... or Wilcoxon rank test.

RESULTS

...t of FK506 on the survival time of MRL-lpr/lpr mice

...order to evaluate the effect of FK506, subcutaneous injection ... times a week) was started on 8-week-old female MRL-lpr/lpr ...ce using high dose (2 mg/kg/day, 8 mice), medium dose (0·8 ...g/kg/day, 7 mice), low dose (0·2 mg/kg/day, 8 mice) or carrier ...vent (for control, 9 mice). As shown in Fig. 1, half of the ...ntrol mice died prior to 25 weeks old. On the other hand, ...arly all mice in the high and medium dose groups survived to ... age. Thus it was concluded the high dose and the medium ... therapy significantly prolonged the survival time of

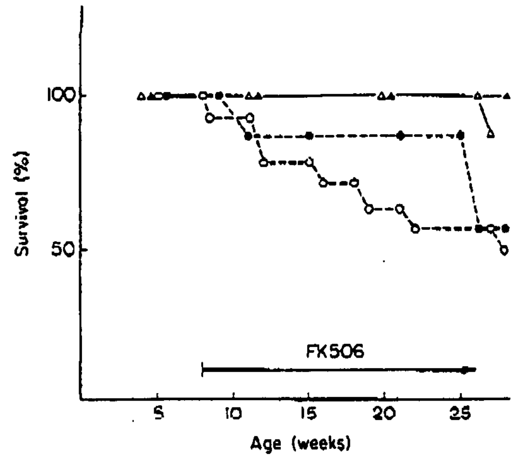

Figure 1. Effect of FK506 on survival of female MRL-lpr/lpr mice. Mice of each group (8–9 mice) were treated with subcutaneous injection 6 times per week (—▲— FK506 2 mg/kg; —△— FK506 0·8 mg/kg; --●-- FK506 0·2 mg/kg; --○-- control).

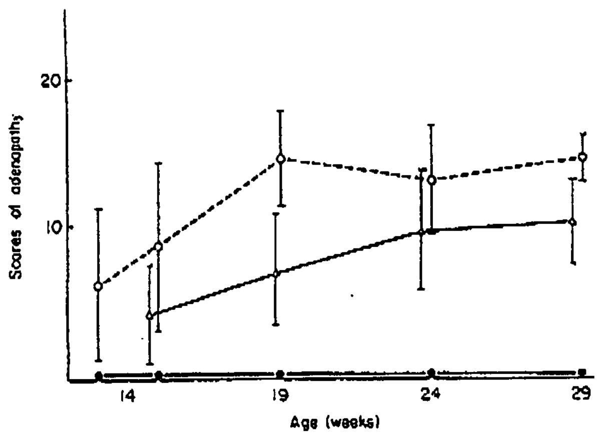

Figure 2. Effect of FK506 on the lymphadenopathy of MRL-lpr/lpr mice (each group consisted of 8–9 mice). The adenopathy was evaluated as scores as described in the Material and Methods (--○-- control; —△— FK506 0·8 mg/kg; —●— FK 506 2 mg/kg).

MRL-lpr/lpr mice compared to the control treatment. The low dose treatment also seemed to have some effect on the survival. At the age of 25 weeks, body weight of mice in each group was as follows: control group, 43·2 ± 3·4 g; low dose group, 43·4 ± 4·9 g; medium dose group, 39·8 ± 2·6 g; high dose group, 36·7 ± 3·2 g.

Effect of FK506 on the lymphadenopathy

The lymphadenopathy was evaluated by scores as described in the Materials and Methods. The placebo-treated mice gradually developed the lymph node swelling (Fig. 2). The high dose treatment of FK506 completely prevented this hyperplasia. Mice treated with the medium dose showed intermediate size of lymph nodes between those of the control and the high dose treatment. The low dose treatment did not have a significant effect on the lymphadenopathy (data not shown).

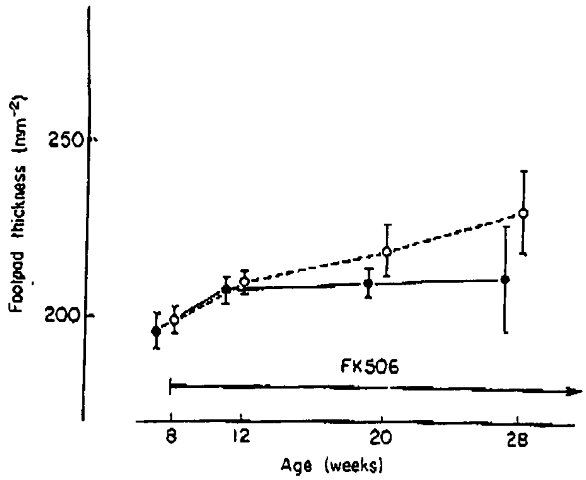

Figure 3. Effect of FK506 on the hind footpad swelling of MRL-lpr/lpr mice (5 mice in each group) (—○-- control; —●— FK506 2 mg/kg).

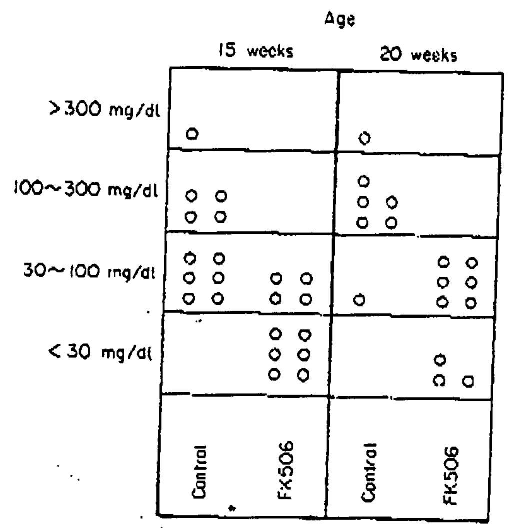

Figure 4. Effect of FK506 on proteinuria. Proteinuria was determined semiquantitatively by colorimetric reaction.

Arthritis

MRL-lpr/lpr mice are also known to develop spontaneous arthritis (Hang, Theofilopoulos & Dixson, 1982). For the evaluation of the effect of FK506 on the arthritis, the thickness of the hind footpad was measured (Fig. 3). FK506 high dose treatment significantly suppressed the swelling of the hind footpad at the age of 28 weeks (Po{W ≧ 38} = 0·016, Wilcoxon rank test).

Effect of FK506 on lupus nephritis and interstitial pneumonitis

Proteinuria was semi-aquantitatively estimated. The high dose treatment suppressed the amounts of proteinuria (Fig. 4). This finding was also confirmed by the histological analysis. Mice treated with high dose FK506 (2 mg/kg/day, 9 mice) and mice of placebo treatment (9 mice) were killed at the age of 4 6 months (3 mice every month) and the histological changes [illegible] compared (Table 1). Figure 5 shows the representative pict[illegible] In the kidney of the control mice, there were marked cell[illegible] infiltrations around arteries and the arterial walls were thick[illegible] ened. The glomerulus were enlarged and lobulated. Hyp[illegible] cellularity in the glomerulus was also recognized. On the contrary the kidneys of FK506-treated mice were found to be nearly normal.

MRL-lpr/lpr mice also develop spontaneous interstitial pneumonitis (Okudaira *et al.*, 1986). The thickening of alveolar septa and the infiltration of mononuclear cells were observed in the lung of control mice. FK506 clearly suppressed the develop[illegible] ment of this pneumonitis (Table 1, Fig. 5).

Serological analysis

Sera were collected individually and the anti-ssDNA and [illegible] dsDNA activities of IgM and IgG classes of each serum [illegible] evaluated by ELISA. Control mice gradually develop IgG [illegible] of anti-ssDNA and anti-dsDNA activities and IgM class of [illegible] dsDNA (Table 2). On the contrary, FK506 high dose treat[illegible] significantly reduced the activities. The medium dose treat[illegible] also seemed to have suppressive effects on the most of anti-DNA activities, even though no statistical significance was obt[illegible] compared to the control. Next, total IgG were evaluated by single radial diffusion plates. The high dose treatment red[illegible] the IgG concentration. Interestingly, the medium dose t[illegible] ment rather enhanced the IgG concentration (Table 3).

Changes of spleen cell phenotypes with FK506 treatment

FK506 dramatically suppressed the hyperplasia of ly[illegible] nodes. Thus, it was rather difficult to obtain a proper amou[illegible] lymph node cells from mice treated with FK506. In accord[illegible] with the effect on the lymph node swelling, the weight of sp[illegible] was significantly reduced by the FK506 treatment (For exam[illegible] at the age of 6 months, control mice had 800 ± 350 mg of sp[illegible] and FK506 mice possessed 250 ± 120 mg of spleen). In ord[illegible] investigate the effect of the treatment on the different cell[illegible] populations, 6-months-old mice were killed and the single [illegible] suspensions from spleen were analysed with monoclonal [illegible] bodies and FACS. Control mice exhibited a high perce[illegible] (42·0 ± 8·5) of double negative T cells (Thy-1.2⁺, Ly[illegible] L3T4⁻), which is characteristic for this mouse. FK506 pred[illegible] inantly suppressed the percentage of this abnormal cell pop[illegible] tion (Table 4).

DISCUSSION

FK506, a newly developed immunosuppressive compound, [illegible] significant effects on the survival time, lymphadenop[illegible] autoantibodies, nephritis, arthritis, interstitial pneumonitis [illegible] the abnormal proliferation of the double negative T ce[illegible] MRL-lpr/lpr mice. These results suggest that this che[illegible] could potentially be used for the treatment of human auto[illegible] mune diseases. It was also shown that FK506 had a signific[illegible] immunosuppressive effect on allograft rejection (Ochiai *et al.*, 1987; Inamura *et al.*, 1988a) and collagen-induced arth[illegible] (Inamura *et al.*, 1988b) in rats.

The similar immunosuppressive effects on MRL-lpr/lpr mice have been reported with Cyclosporin A (Smith, Chus[illegible]

Table 1. Histological evaluation of kidney and lung from FK506-treated MRL-lpr/lpr mice

Age	4 months		5 months		6 months	
Group*	Control	FK506	Control	FK506	Control	FK506
Kidney						
Cell numbers in one glomerulus	69±10	39±8†	62±15	40±7†	88±32	50±17†
Perivascular infiltration	++‡	−∼±	++∼+++	−∼+	+++	−∼+
Extra capillary crescent	+	−	++	−	++∼+++	−
Lung						
Thickening of alveolar septae	−	−	++	+	++	+
Infiltration of mononuclear cells in alveolar septae	+	−	++	+	++	+
Peribronchial and perivascular lymph follicles	−	−	+	−	++	+
Germinal centers	−	−	+	−	+	−

*Each group consisted of three mice. FK506 dose was 2 mg/kg per day.
†$P<0{\cdot}005$.
‡Arbitrary scale of histological findings: −, not observed; +, mild; ++, moderate; +++, severe changes.

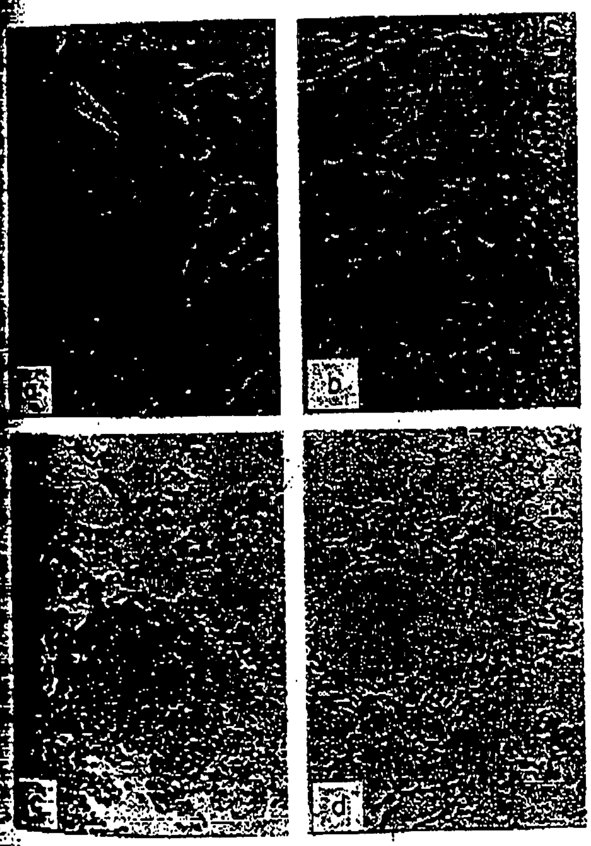

Figure 5. Representative kidney and lung sections from FK506 or placebo-treated MRL-lpr/lpr mice. (a) Kidney from placebo-treated 6-month-old mice (×65). (b) Kidney from FK506 (2-mg/kg/day)-treated 6-month-old mice (×130). (c) Lung from placebo-treated 5-month-old mice (×130). (d) Lung from FK506 (2 mg/kg/day)-treated 5-month-old mice (×130).

Table 2. Effect of FK506 on anti-DNA antibodies in MRL-lpr/lpr mice

		Anti-ssDNA (units)*		Anti-dsDNA (units)*	
Age	Group†	IgM	IgG	IgM	IgG
3 months	Control	660±364	314±216	32±11	51±10
	Medium dose**	244±213	ND‡	70±57	ND
	High dose**	86±21§	8±4§	18±20	13±5¶
6 months	Control	369±111	476±229	363±132	362±243
	Medium dose	353±90	294±274	218±139	175±169
	High dose	117±55¶	37±35§	9±5¶	6±1§

*One thousand units are the anti-DNA activity of each class in the pooled standard serum from old MRL-lpr/lpr mice.
†Each group consisted of six or seven samples
‡ND, not determined; §$P<0{\cdot}01$; ¶$P<0{\cdot}005$.
**High dose was 2 mg/kg/day; medium dose was 0·8 mg/kg/day.

Table 3. Effect of FK506 on serum IgG concentrations (mg/ml) in MRL-lpr/lpr mice*

Age	5 months	7 months
Control	13·7±2·8	17·7±6·8
Low dose†	17·3±1·5	ND‡
Medium dose†	29·0±2·8§	33·6±7·4**
High dose†	5·2±2·4¶	4·8±0·5¶

*Four to six mice per group were tested individually by radial immunodiffusion plates.
†High dose was 2 mg/kg; medium dose was 0·8 mg/kg; low dose was 0·2 mg/kg.
‡ND, not determined; §$P<0{\cdot}005$; ¶$P<0{\cdot}01$; **$P<0{\cdot}05$.

Table 4. Surface phenotypes of spleen cells from FK506 treated MRL-lpr/lpr mice*

Antigen	B220⁺	Thy-1.2⁺	Lyt-2⁺	L3T4⁺	Thy-1.2⁺, Lyt-2⁻, L3T4⁻
Control	55·2±1·3	68·7±8·0	8·5±1·9	18·2±3·6	42·0±8·5
FK506†	49·3±2·7	40·4±12·5	10·7±2·4	20·6±5·5	9·1±4·7‡

*Six-month-old mice (3 mice in each group) were killed and the spleen cell suspensions were analysed by FACS after staining with FITC-conjugated monoclonal antibodies. Values were positive percentage of the stained cells.

†The dose was 2 mg/kg/day.

‡$P<0·01$.

Steinberg, 1984; Isenberg *et al.*, 1981, Okudaira *et al.*, 1986; Mountz *et al.*, 1987). However, there are some differences. firstly, the dose of FK506 was $\frac{1}{10}$ to $\frac{1}{15}$ lower than that of Cyclosporin A. Secondly, our data clearly demonstrated that FK506 suppressed anti-ssDNA and dsDNA antibodies. On the other hand, recent data with Cyclosporin A showed that the anti-DNA antibodies were not affected by the treatment (Mountz *et al.*, 1987). Therefore, Mountz *et al.* pointed out, anti-DNA antibodies might have no relation to the immunopathology because Cyclosporin A nevertheless decreased the pathological changes in arthritis and glomerulonephritis. However, there is ample evidence supporting the relationship of anti-DNA antibodies and lupus nephritis (Borel *et al.*, 1978; Dixon *et al.*, 1983; Yoshida *et al.*, 1981; Brunea & Benveniste, 1979). In this regard, FK506 could be more effective on these autoimmune mice because it suppressed T-cell and B-cell immunity. The difference of IgG concentration in the sera from the high dose treated and the medium dose treated mice is interesting. Although the reduced IgG with the high dose treatment is clear, the elevated IgG with the medium dose treatment could be in part affected by the reduced rheumatoid factor by the treatment. This needs further investigation.

There were some differences in the body weight of mice between the control group and high dose group. It is known that MRL-lpr/lpr mice abnormally gain weight perhaps due to the hyperplasia in the lymphoid organs, edema and some other mechanisms. Thus, it is possible to consider this body weight loss as a therapeutic effect of FK506. In fact, the high dose treated mice looked to be very healthy and active compared to the control mice at the same age. On the contrary, it is also possible that this body weight loss could be due to a side-effect (Inamura *et al.*, 1988b). Recent experiments suggested that low calorie intake prolonged the life span of MRL-lpr/lpr mice and reduced lympho-proliferation (Beach, Gershwin & Hurley, 1982; Kubo, Day & Good, 1984). However, it is extremely unlikely that the effects of FK506 on MRL/lpr mice could only be due to the food intake restriction. For example, calorie intake did not significantly reduce anti-DNA antibodies. Further studies should be carried out in order to know the possibilities of the side effects. However, at the present time, FK506 seems to be a quite promising drug for the treatment of autoimmune diseases.

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Calcineurin Is a Common Target of Cyclophilin-Cyclosporin A and FKBP-FK506 Complexes

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Summary

Although the immediate receptors (immunophilins) of the immunosuppressants cyclosporin A (CsA) and FK506 are distinct, their similar mechanisms of inhibition of cell signaling suggest that their associated immunophilin complexes interact with a common target. We report here that the complexes cyclophilin-CsA and FKBP-FK506 (but not cyclophilin, FKBP, FKBP-rapamycin, or FKBP-506BD) competitively bind to and inhibit the Ca^{2+}- and calmodulin-dependent phosphatase calcineurin, although the binding and inhibition of calcineurin do not require calmodulin. These results suggest that calcineurin is involved in a common step associated with T cell receptor and IgE receptor signaling pathways and that cyclophilin and FKBP mediate the actions of CsA and FK506, respectively, by forming drug-dependent complexes with and altering the activity of calcineurin-calmodulin.

Introduction

Complexes of immunophilins (immunosuppressant-binding proteins) and their immunosuppressive ligands are proving to be valuable reagents for probing signal transduction pathways (Schreiber, 1991). Studies of T cell receptor (TCR)–mediated transcription and IgE receptor–mediated exocytosis with the immunosuppressants cyclosporin A (CsA) and FK506 suggest the existence of a common cytoplasmic step in these Ca^{2+}-dependent signaling pathways (Hultsch et al., 1991). Rapamycin, an immunosuppressant structurally related to FK506, appears to act upon a similar step that exists in distinct signaling pathways associated with growth factor receptors (Dumont et al., 1990a).

The biological properties of CsA and FK506 are remarkably similar (Johansson and Moller, 1990; Lin et al., 1991). Aside from differences in potency, the only distinction between these two compounds in studies of their actions in T cells and mast cells is their response to rapamycin (Dumont et al., 1990b; Bierer et al., 1990a; Hultsch et al., 1991) and 506BD (Bierer et al., 1990b; Hultsch et al., 1991). These latter agents block the actions of FK506, but not CsA, in T cells and mast cells. FK506, but not CsA, is able to block the actions of rapamycin in T cells (Dumont et al., 1990b; Bierer et al., 1990a). These results suggest that distinct immunophilins are responsible for mediating the actions of CsA and FK506, and a common immunophilin may be responsible for mediating the actions of both FK506 and rapamycin. Much evidence has accumulated that supports roles for cyclophilin–CsA, FKBP–FK506, and FKBP–rapamycin complexes in the inhibition of the aforementioned processes, although the relevant cyclophilin and FKBP family members have not been uniquely defined in most cases. The similarities in all other aspects of CsA and FK506 function suggest that their associated immunophilin complexes are acting upon a (possibly immediate) common biological target molecule that is not a target of the FKBP–rapamycin complex, free FKBP, or free cyclophilin.

We now report the competitive binding of a human cyclophilin A (or murine cyclophilin C)–CsA complex and a human FKBP12–FK506 complex to a common cellular target that is not bound by either cyclophilin A (or C), FKBP12, or FKBP12–rapamycin. The target is shown to be the Ca^{2+}- and calmodulin-dependent serine/threonine phosphatase calcineurin (Klee and Krinks, 1978; Klee et al., 1979; Stewart et al., 1982), as well as a complex of calcineurin and calmodulin. Calcineurin meets the biochemical requirements of the common target of immunophilin–drug complexes and thus is a potential component of TCR and IgE receptor signaling pathways involved in transcription and exocytosis.

Results

A Common Set of Proteins Bind to Cyclophilin-CsA and FKBP-FK506, but Not Cyclophilin, FKBP, CsA, FK506, Rapamycin, or FKBP-Rapamycin

A chimeric gene encoding a glutathione S-transferase–FKBP12 fusion protein (GFK) was constructed by fusing the cDNA encoding FKBP12 to that encoding the carboxyl terminus of glutathione S-transferase (Smith and Johnson, 1988). After transformation of the resulting construct, pGFK12, into Escherichia coli XA90, induction with isopropyl-β-D-thiogalactopyranoside (IPTG) yielded the fusion protein GFK as the major constituent of soluble, cellular proteins. GFK was partially purified by ammonium sulfate fractionation (40%–80%), glutathione affinity chromatography, and DE-52 anion exchange chromatography as detailed in Experimental Procedures. GFK fractions after DE-52 chromatography were nearly homogeneous as judged by Coomassie blue staining. However, several other protein bands were visible with silver staining (Figure 1, lane 1).

The rotamase activity of GFK and its ability to be inhibited by FK506 and rapamycin were determined in the presence and absence of reduced glutathione. GFK has rotamase activity and affinities for FK506 and rapamycin similar to those of recombinant human FKBP12; furthermore, neither its rotamase activity nor affinities for the

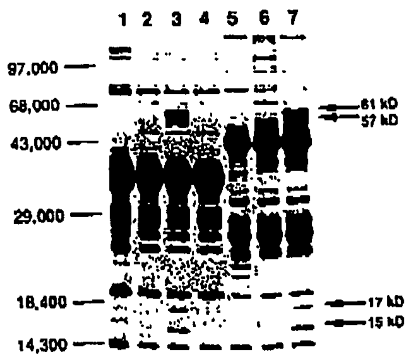

Figure 1. Detection of a Common Set of Proteins from Calf Brain Extract That Bind to GFK–FK506 and GCyP–CsA, But Not GFK, GCyP, or GFK–Rapamycin

The fusion proteins GFK and GCyP, with or without drugs, were incubated with calf brain extract. Glutathione–Sepharose was then added to adsorb the fusion proteins and associated target proteins. After three washes with the incubation buffer and 0.2% Triton X-100, the glutathione–Sepharose was resuspended in SDS sample loading buffer, heated in boiling water, and subjected to 12% SDS–polyacrylamide gel electrophoresis. The gel was silver stained. Lane 1, GFK control; lane 2, GFK plus extract; lane 3, GFK–FK506 plus extract; lane 4, GFK–rapamycin plus extract; lane 5, GCyP control; lane 6, GCyP plus extract; lane 7, GCyP–CsA plus extract.

drugs are affected by the presence of glutathione (data not shown). Similar results were obtained with a glutathione S-transferase–cyclophilin C fusion protein, GCyP, described in the accompanying paper (Friedman and Weissman, 1991). Thus, it appears that immunophilin and glutathione S-transferase domains act independently in the fusion protein.

Since it is known that both CsA and FK506 inhibit Ca^{2+}-dependent signaling pathways (Lin et al., 1991), Ca^{2+} and Mg^{2+} were included in the incubation buffer (see Experimental Procedures). Under these conditions, proteins of M_r 61,000, 57,000, 17,000, and 15,000 from a calf thymus extract were found to be specifically adsorbed by the GFK–FK506 and GCyP–CsA complexes, but not by GFK or GCyP alone or the GFK–rapamycin complex (see Figure 1). Furthermore, the same set of proteins was detected in other tissues such as bovine brain and spleen, with brain being the most reliable source.

Competitive binding experiments were carried out with recombinant FKBP12 and cyclophilin A and their respective drug complexes. After the set of four target proteins was adsorbed onto the glutathione–Sepharose affinity matrix, elution was attempted with immunophilins, the drugs, or the immunophilin–drug complexes. As shown in Figure 2, the set of four proteins can be eluted from a GFK–FK506 affinity matrix with both recombinant FKBP12–FK506 and recombinant cyclophilin A–CsA complexes. In contrast, these proteins are not eluted by free immunophilins or unbound drugs. In addition, the FKBP12–rapamycin complex does not elute the target proteins (Figure 2), in agreement with previous observations (Figure 1, lane 4). The ability of both cyclophilins A and C, when complexed

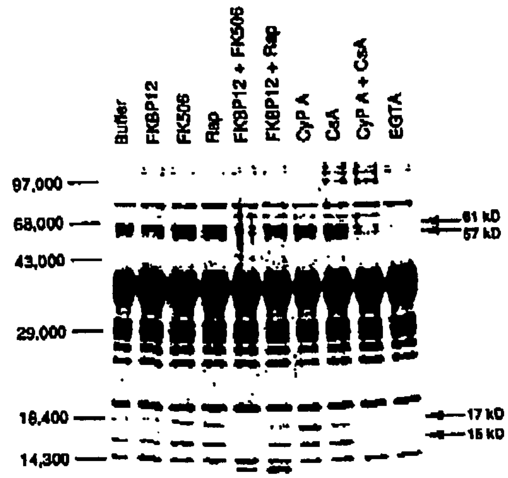

Figure 2. In Vitro Competition Experiments with Recombinant FKBP12, Cyclophilin A, Individual Drugs, Immunophilin–Drug Complexes, and EGTA

The four target proteins were first adsorbed onto glutathione–Sepharose using GFK–FK506 as shown in Figure 1, lane 3. The immobilized GFK–FK506–target protein complex was then incubated with the following: lane 1, buffer (as control); lane 2, recombinant human FKBP12; lane 3, FK506; lane 4, rapamycin; lane 5, FKBP12–FK506; lane 6, FKBP12–rapamycin; lane 7, recombinant human cyclophilin A; lane 8, CsA; lane 9, cyclophilin A–CsA; and lane 10, EGTA. The glutathione–Sepharose with adsorbed GFK–FK506 and bound target proteins was precipitated by centrifugation and washed twice before being subjected to 12% SDS-PAGE and silver staining. The 12 kd protein in lanes 2, 5, and 6 corresponds to FKBP12 from the incubation.

to CsA, to interact with the same set of proteins is noteworthy, as discussed below.

Divalent Metal Ion Dependence of Immunophilin Drug–Target Protein Complex Formation, and Purification of the Target Proteins by EGTA Elution

When Ca^{2+} and Mg^{2+} were omitted from the incubation buffer, no target proteins were retained by either GFK–FK506 or GCyP–CsA. To further test the importance of divalent metal ions for interaction between immunophilin–drug complexes and target proteins, the adsorption experiment was performed in the presence of the Ca^{2+} chelator EGTA. GFK–FK506 and GCyP–CsA complexes no longer retained the target proteins when EGTA was present. In addition, EGTA was found to be capable of eluting the four proteins from the GFK–FK506 complex immobilized on glutathione–Sepharose without a significant effect on the interactions between GFK and glutathione–Sepharose (Figure 2, lane 10).

GFK–FK506 bound to glutathione–Sepharose matrix was loaded with calf thymus extract and eluted with EGTA; analysis of the eluate is shown in Figure 3 (lanes 3 and 6). Two contaminant proteins coelute with the four target proteins, the 38 kd GFK and a less abundant M_r 26,000 protein that may be a glutathione S-transferase either from

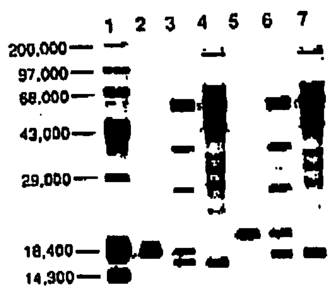

Figure 3. Ca^{2+}-Dependent Gel Mobility Shift of Calmodulin, the 17 kd and 15 kd EGTA-Eluted Target Proteins, and Calcineurin B

Standard calmodulin (from Sigma; 0.25 μg), EGTA eluate from calf thymus (2 μg), and standard calcineurin (from Sigma; 3 μg) were subjected to 12% SDS–PAGE in the presence of 5 mM $CaCl_2$ or 5 mM EGTA, followed by silver staining. Lane 1, molecular weight standards; lane 2, calmodulin plus Ca^{2+}; lane 3, EGTA eluate plus Ca^{2+}; lane 4, calcineurin plus Ca^{2+}; lane 5, calmodulin plus EGTA; lane 6, EGTA eluate plus EGTA; lane 7, calcineurin plus EGTA.

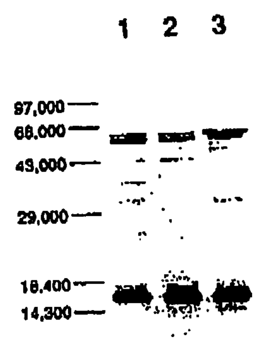

Figure 4. Western Blot Analysis of EGTA Eluate with Anti-Calcineurin Antibodies

EGTA-eluted target proteins (lane 1, 2 μg of total protein as used in Figure 3, lanes 3 and 6), calf brain calcineurin from Sigma (lane 2, 0.5 μg), and purified calf brain calcineurin (lane 3, 0.6 μg provided by Dr. C. B. Klee) were subjected to 12% SDS–PAGE and electroblotted onto nitrocellulose, which was then developed with rabbit anti-calcineurin IgG and goat anti-rabbit IgG conjugated with alkaline phosphatase.

the proteolytic cleavage of GFK or from the calf thymus extract. With this procedure, over 40 μg of proteins can be purified from 20 ml of calf thymus extract (120 mg of protein).

Identification of the M_r 17,000 Protein as Calmodulin, the M_r 61,000 and 57,000 Proteins as Calcineurin A, and the M_r 15,000 Protein as Calcineurin B

Since the binding of the target proteins to the immunophilin–drug complexes is metal ion (especially Ca^{2+}) dependent, we speculated that the M_r 17,000 protein was calmodulin. One of the distinctive properties of calmodulin is its Ca^{2+}-dependent gel mobility shift; i.e., calmodulin migrates faster in the presence of Ca^{2+} during SDS–polyacrylamide gel electrophoresis (Klee et al., 1979). Indeed, when the EGTA eluate was subjected to SDS–PAGE beside a calmodulin standard (Sigma), the M_r 17,000 protein bands exhibited the Ca^{2+}-dependent gel mobility shift characteristic of calmodulin (Figure 3, lanes 2 and 3 versus 5 and 6). That the M_r 17,000 protein is calmodulin was confirmed in subsequent experiments with authentic calmodulin.

We reasoned that the remaining three proteins could be part of a multisubunit complex of calmodulin-binding proteins, such as a Ca^{2+}- and calmodulin-dependent kinase or Ca^{2+}- and calmodulin-dependent phosphatase. Calcineurin (Klee et al., 1988) is composed of two subunits, calcineurin A, a 61 kd polypeptide, and calcineurin B, a 19 kd polypeptide. As the 19 kd calcineurin B migrates at about 15 kd on SDS–PAGE, we considered that the M_r 61,000 and 15,000 proteins are calcineurin A and B, respectively. It was also known that calcineurin A undergoes proteolysis to yield a 57 kd protein.

When the four target protein bands were blotted onto polyvinylidene difluoride membranes and subjected to N-terminal sequencing, they were all found to be N-terminally blocked (not shown). This is in agreement with the fact that both subunits of calcineurin, the 57 kd proteolytic fragment of calcineurin A, and calmodulin have covalent modifications of their N-termini (Klee et al., 1988; Aitken et al., 1984; Klee and Vanaman, 1982).

Calcineurin B is a calcium-binding protein with four Ca^{2+}-binding EF hands (Aitken et al., 1984); like calmodulin, it exhibits a gel mobility shift in the presence of calcium during SDS–PAGE. The M_r 15,000 EGTA-eluted protein did migrate faster in the presence of calcium (Figure 3, lanes 3 and 6). It was also found to comigrate with a calcineurin B standard (Sigma) in the presence or absence of calcium (Figure 3, lanes 4 and 7). The M_r 61,000 and M_r 57,000 EGTA-eluted proteins comigrate with a calcineurin A standard (Sigma); these proteins do not undergo a gel mobility shift in the presence of Ca^{2+}.

A Western blot of the EGTA eluate from calf thymus with polyclonal antibodies against bovine brain calcineurin helped to establish that the M_r 61,000, 57,000, and 15,000 proteins are calcineurin A, a proteolytic fragment of calcineurin A, and calcineurin B, respectively (Figure 4). A $^{45}Ca^{2+}$ ligand-blotting experiment with the EGTA eluate further supports the identity of the 15 kd protein as calcineurin B (Figure 5). The weaker response of calmodulin (M_r 17,000) was anticipated, as it is known that under the same blotting conditions calmodulin provides a weaker signal (C. B. Klee, personal communication). The identification of the retained proteins was next confirmed by experiments with authentic samples of calcineurin and calmodulin.

Calcineurin Binds to FKBP–FK506 and Cyclophilin–CsA in a Ca^{2+}-Dependent Fashion, and Its Phosphatase Activity toward a Phosphopeptide Substrate Is Specifically Inhibited by the Two Immunophilin–Drug Complexes

The identification of calcineurin as a common target of immunophilin–drug complexes was confirmed by experi-

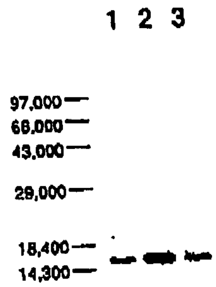

Figure 5. ⁴⁵Ca^{2+} Ligand Blotting of Calcineurin B

Lane 1, EGTA-eluted target proteins from calf thymus (2 μg of total proteins); lane 2, purified calf brain calcineurin (1 μg, provided by Dr. C. B. Klee); lane 3, calf brain calcineurin from Sigma (1 μg).

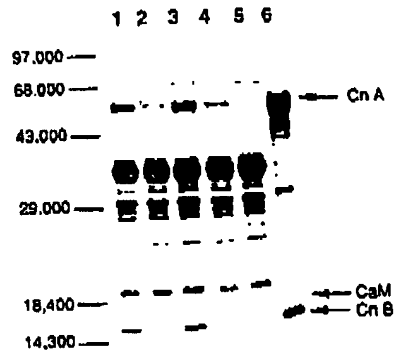

Figure 6. Glutathione–Sepharose Adsorption Experiments Using Bovine Brain Calcineurin and GFK–FK506

The experiments were carried out in the same way as described in the legend to Figure 1, except 2 μg of authentic calf brain calcineurin was used in place of the calf brain extract. The incubation buffer contained 50 mM Tris–HCl (pH 7.5), 100 mM NaCl, 2 mM $MgCl_2$, 2 mM $CaCl_2$ (buffer A) or 50 mM Tris–HCl (pH 7.5), 100 mM NaCl (buffer B). For each adsorption experiment, the final concentrations of GFK and FK506 were 2 μM and 20 μM, respectively. Lane 1, calcineurin with buffer A; lane 2, calcineurin with buffer B and 20 mM EGTA; lane 3, calcineurin with buffer A and 1 μM calmodulin; lane 4, calcineurin with buffer B, 1 μM calmodulin, and 20 mM EGTA; lane 5, 1 μM calmodulin in buffer A; lane 6, 2 μg of bovine brain calcineurin used in experiments shown in lanes 1–5. CaM = calmodulin; CnA = calcineurin A; CnB = calcineurin B.

ments with authentic calcineurin purified from bovine brain. Calcineurin was found to be retained by GFK–FK506 and GCyP–CsA, but not by GFK, GCyP, or GFK–rapamycin. The GFK–FK506 complex binds directly to calcineurin in the presence of Ca^{2+} without calmodulin (Figure 6, lane 1), and the binding is dependent on Ca^{2+} and Mg^{2+} (lane 2). In the presence of calmodulin, however, increased amounts of calcineurin (both A and B subunits) appear to be adsorbed by the same amount of GFK–FK506 complex (Figure 6, lane 3), and calmodulin is retained as well. The binding of calcineurin–calmodulin by GFK–FK506 was abolished by EGTA (lane 4). Calmodulin alone does not bind to the GFK–FK506 complex in the presence of calcium (lane 5). These experiments demonstrate that the immunophilin–drug complexes bind directly to calcineurin and only indirectly, via calcineurin, to calmodulin.

Calcineurin is known to be a Ca^{2+}- and calmodulin-dependent protein phosphatase (Stewart et al., 1982). Using a phosphorylated peptide fragment from the regulatory subunit of cAMP-dependent kinase as a substrate, the phosphatase activity of authentic calcineurin was assayed in the presence of the immunophilins, the individual drugs, and their respective complexes with or without calmodulin. As shown in Figures 7A and 7B, both the intrinsic Ca^{2+}-dependent and Ca^{2+}- and calmodulin-stimulated phosphatase activities of calcineurin are potently inhibited by FKBP12–FK506 and cyclophilin A–CsA complexes, in agreement with the glutathione–Sepharose adsorption experiments (Figure 6). It is worth noting that complexes of FKBP–rapamycin and FKBP–506BD do not significantly inhibit the phosphatase activity (Figures 7A and 7B), in full agreement with the competitive binding assay (Figure 2) and the previous observations that rapamycin inhibits different, calcium-independent signaling pathways (Dumont et al., 1990b) and that 506BD, although a potent rotamase inhibitor of FKBP12, is a very weak inhibitor of TCR-mediated signaling (Bierer et al., 1990b).

Discussion

CsA and FK506, despite their lack of structural similarities, have highly similar biological properties. Both molecules interfere with the TCR-mediated signaling pathway that results in the transcription of early T cell activation genes, although FK506 is able to do so at 100-fold lower concentrations (Tocci et al., 1989). The transcriptional activation and (to a lesser degree) DNA-binding properties of the transcription factors NF-AT, AP-3, and (partially) NF-κB are potently inhibited by both immunosuppressants (Emmel et al., 1989; Mattila et al., 1990). Their site of action has been localized to the cytoplasm: they act following early membrane-associated events but prior to nuclear events. Without interfering with membrane-associated signaling processes, both molecules inhibit an IgE receptor–mediated signaling pathway that results in the exocytosis of secretory granules in mast cells (Hultsch et al., 1991). This cytoplasmic event is sensitive to the actions of CsA and FK506 at concentrations similar to those required to inhibit transcription in T cells. A common feature of CsA- and FK506-sensitive signaling pathways identified to date is their dependence on Ca^{2+}; conversely, several Ca^{2+}-independent signaling pathways have been studied that are resistant to the actions of CsA and FK506 (Lin et al., 1991).

In addition to their similar effects on cell function, both CsA and FK506 bind with high affinity to abundant cytosolic receptors (immunophilins) that catalyze the interconversion of cis- and trans-rotamers of peptidyl-prolyl amide bonds of peptide and protein substrates. The rotamase activity of cyclophilin A (CsA-binding protein; the "A" descriptor refers to the originally discovered isoform [Handschumacher et al., 1984], whereas cyclophilin C refers to

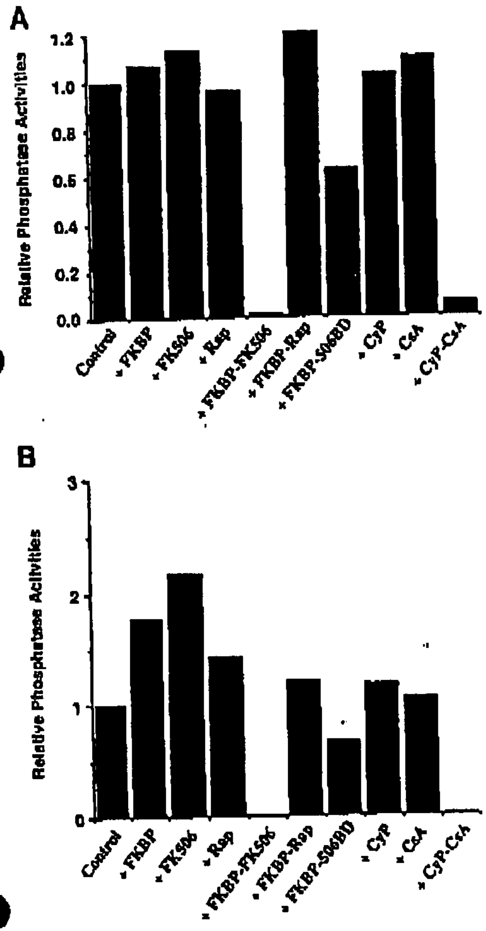

Figure 7. Inhibition of Ca^{2+}- or Ca^{2+}- and Calmodulin-Dependent Phosphatase Activity of Calcineurin toward a Phosphopeptide Substrate by FKBP12–FK506 and Cyclophilin A–CsA Complexes

Under the assay conditions (see Experimental Procedures), the amounts (cpm) of $^{32}P_i$ released from the phosphopeptide were taken as a measure of phosphatase activity without further conversion into initial velocities of the reactions. The final concentrations for different components were as follows: 40 nM calcineurin; 200 nM each FKBP12 and cyclophilin A; 300 nM each FK506, rapamycin, and CsA; and 2.3 µM 506BD. (A) Phosphatase assay in the presence of calmodulin (60 nM). (B) Phosphatase assay in the absence of calmodulin. The presence of immunophilins, their ligands, and the corresponding complexes is indicated below the bars.

the isoform described by Friedman and Weissman [1991]) is inhibited by CsA (K_i = 6 nM) (Fischer et al., 1989; Takahashi et al., 1989). The rotamase activity of the FK506- and rapamycin-binding protein FKBP12 (the "12" descriptor refers to the originally discovered 12 kd isoform [Siekierka et al., 1989; Harding et al., 1989]; other isoforms [Fretz et al., 1991], including the 13 kd FKBP13 [Jin et al., 1991], have been described) is inhibited by FK506 (K_i = 0.4 nM) and rapamycin (K_i = 0.2 nM) (Bierer et al., 1990a). Although sequence-related members of both the cyclophilin and FKBP families exist, there are no similarities in sequence between the two families (Standaert et al., 1990). The atomic structure of cyclophilin A has not been reported, but a preliminary investigation (J. Kallen, M. D. Walkinshaw, and M. Zurini, personal communication) suggests its structure differs significantly from the recently reported atomic structures of FKBP12 (Michnick et al., 1991; Moore et al., 1991) and the FKBP12–FK506 complex (Van Duyne et al., 1991).

Recent investigations have shown that the inhibition of the rotamase activity of immunophilins is an insufficient requirement for mediating the actions of immunosuppressants (Bierer et al., 1990a). For example, a potent, nonnatural rotamase inhibitor (506BD, which inhibits the rotamase action of FKBP12 with K_i = 5 nM) does not inhibit TCR or IgE receptor–mediated signaling pathways at low concentrations (Bierer et al., 1990b). On the other hand, 506BD was found to potently inhibit the actions of both FK506 and rapamycin. Similar results have recently been reported with cyclophilin ligands (Sigal et al., 1991). Binding of immunosuppressant to immunophilin results in a gain, rather than a loss, of function. This is evident in genetic studies in Saccharomyces cerevisiae that provide strong support for a role for immunophilin–drug complexes. For example, deletion of yeast FKBP12 results in rapamycin-resistant strains that are returned to rapamycin sensitivity following transfection of human FKBP12 (Koltin et al., 1991; Heitman et al., 1991b). Related results were obtained with cyclophilin A and CsA (Tropschug et al., 1989) and FKBP12 and FK506 (Heitman et al., 1991a). The role of immunophilins in the absence of drug is an active area of investigation: Friedman and Weissman (1991) describe the association of an immunophilin (cyclophilin C) with specific target proteins, and a cyclophilin variant has been shown to be necessary for the proper trafficking of rhodopsin in Drosophila (Stamnes et al., 1991). The immunophilin ligands CsA, FK506, and rapamycin are equipped with structural elements that provide for high affinity interactions with their immunophilin receptors (in the case of FK506 and rapamycin, these FKBP-binding elements are very similar) and effector elements that, when presented by immunophilin, determine the target (Bierer et al., 1990a, 1990b).

The difference in response to rapamycin and 506BD distinguishes the otherwise inseparable biological actions of CsA and FK506, suggesting that their immediate receptors are distinct (cyclophilin and FKBP) but that these complexes may eventually converge on a common target. The simplest possibility is that the common target binds directly to both the cyclophilin–CsA and FKBP–FK506 complexes. The experiments reported herein identify calcineurin, a Ca^{2+}- and calmodulin-dependent serine/threonine phosphatase (Klee and Krinks, 1978; Klee et al., 1979; Stewart et al., 1982) previously shown to be the predominant calmodulin-binding protein in T lymphocytes (Kincaid et al., 1987), as a common target of these immunophilin–drug complexes.

Calcineurin, which is a complex of 61 kd calcineurin A and 19 kd calcineurin B, and calmodulin were retained on a cyclophilin C or FKBP12-based matrix only when it had been preloaded with CsA or FK506, respectively (Figure

1). The observation that CsA is required for the cyclophilin C matrix to retain the calcineurin A is in accord with the results of Friedman and Weissman (1991). The additional protein of 57 kd retained from the tissue extracts used in these studies is very likely the known proteolytic fragment resulting from cleavage of the C-terminus of calcineurin A (Hubbard and Klee, 1989). Affinity matrices based on cyclophilin C, FKBP12, FKBP12–rapamycin (Figure 1), CsA, FK506, and rapamycin (Fretz et al., 1991) do not retain any of these proteins. Elution of the proteins from either immunophilin–drug matrix was also achieved with soluble cyclophilin A–CsA or FKBP12–FK506 complexes (Figure 2). These results suggest the two immunophilin–drug complexes bind competitively to calcineurin and also indicate that two different forms of cyclophilin (A and C) are able to present CsA to the common target. The Ca^{2+}-dependent binding of the immunophilin–drug complexes to calcineurin is in keeping with the correlation of CsA and FK506 actions on Ca^{2+}-dependent signaling pathways (Figure 2).

Treatment of the eluted proteins with Ca^{2+} prior to gel electrophoresis resulted in a gel mobility shift for the M_r 15,000 and 17,000 bands that is characteristic of myristoylated calcineurin B and calmodulin (Klee et al., 1979), respectively (Figure 3). Blotting experiments with anti-calcineurin antibodies (Figure 4) and $^{45}Ca^{2+}$ (Figure 5) provide further support for the identification of the M_r 61,000, 57,000, 17,000, and 15,000 proteins as calcineurin A, the C-terminal peptide cleavage product of calcineurin A, calmodulin, and calcineurin B, respectively. In addition, the primary immunophilin–drug interaction site within the target calcineurin–calmodulin complex was shown to reside within calcineurin by affinity experiments with authentic calcineurin samples that lacked calmodulin. In these experiments only the cyclophilin C–CsA and FKBP–FK506 matrices are able to retain authentic calcineurin (Figure 6). Finally, after the initial review of this article, 25 and 20 amino acid fragments derived from the M_r 61,000 and 15,000 proteins obtained from the affinity experiments described above were sequenced by automated Edman degradation and shown to be 100% identical to sequences in calcineurin A (SQTTGFPSLITIFSAPNYLDVYNNK) and calcineurin B (IYDMDKDGYISNGELFQVLK), respectively.

The influence of immunophilins or immunophilin–drug complexes on the phosphatase activity of authentic calcineurin in the presence of Ca^{2+} and calmodulin was assayed with both p-nitrophenyl phosphate and a phosphopeptide substrate (H_2N-Asp-Leu-Asp-Val-Pro-Ile-Pro-Gly-Arg-Phe-Asp-Arg-Arg-Val-Ser(OPO_3^{2-})-Val-Ala-Ala-Glu-CO_2H). In accord with the binding studies, a specific effect is seen with the complexes of both cyclophilin A–CsA and FKBP12–FK506. Whereas these complexes induce a slight increase in the phosphatase activity of calcineurin–Ca^{2+}–calmodulin (by a factor of ~2–3) toward p-nitrophenyl phosphate (data not shown), they potently inhibit activity toward the phosphopeptide substrate in the presence (Figure 7A) or absence (Figure 7B) of calmodulin. These results suggest that the biological function of the immunophilin–drug complexes may be to inhibit the phosphatase activity of calcineurin, but that this may be achieved by binding to a site adjacent to the active site, rather than to the active site. The small p-nitrophenyl phosphate reagent presumably interacts with calcineurin nearly exclusively at active-site residues, whereas the phosphopeptide, which is composed of a sequence derived from the phosphorylation site on the RII subunit of cAMP-dependent protein kinase (a calcineurin substrate) (Blumenthal et al., 1986), is presumed to make extensive contact with the enzyme. In both phosphatase assays, little or no effect was observed with cyclophilin A, FKBP12, CsA, FK506, or rapamycin alone, or with the FKBP12–rapamycin and FKBP12–506BD complexes (Figures 7A and 7B).

Whereas an affinity matrix based on cyclophilin C–CsA was used in the initial adsorption protocol, recombinant cyclophilin A–CsA was found to elute calcineurin–calmodulin from the FKBP12–FK506 affinity matrix and to inhibit the phosphatase activity of calcineurin. Although the relative affinity of different cyclophilin–CsA complexes is unknown, these preliminary studies provide evidence for redundancy in the cyclophilin component. The ability of cyclophilin C to participate in calcineurin binding supports the possibility that other cyclophilin isoforms mediate the actions of CsA (Friedman and Weissman, 1991; Price et al., 1991).

These biochemical investigations suggest that the Ca^{2+}- and calmodulin-dependent phosphatase calcineurin is a common "downstream" biological target of CsA and FK506. As these agents exhibit specificity for activation pathways that induce an increase in intracellular Ca^{2+} concentration, such as those mediated by the TCR and the IgE receptor, calcineurin may be involved in regulating the phosphorylation state of a downstream component of these signaling pathways. The cellular specificity of the actions of CsA and FK506 may be related to their selective interactions with specific isoforms of calcineurin or due to the existence of cell-specific calcineurin substrates.

The competitive binding of cyclophilin A–CsA and FKBP12–FK506 to calcineurin is surprising in light of the absence of apparent structural similarities between the immunophilins cyclophilin A and FKBP12 and their ligands CsA and FK506. It is possible that different binding elements within the same binding site on calcineurin–calmodulin are used by these distinct immunophilin–drug complexes. Although the final resolution of this dilemma may require detailed structural analyses of multimeric ensembles, recent progress in the determination of the structure of immunophilins (Michnick et al., 1991; Moore et al., 1991) and their drug complexes (Van Duyne et al., 1991) and in the understanding of the domain structure of calcineurin (Hubbard and Klee, 1989), together with the availability of genetic systems that lack specific immunophilins (and thus present the characteristic drug-resistance phenotype) (Koltin et al., 1991; Heitman et al., 1991a, 1991b), provides significant opportunities for research in this area.

Our studies define the Ca^{2+}- and calmodulin-dependent phosphatase calcineurin as a common and specific target of cyclophilin–CsA and FKBP–FK506 complexes in vitro. Biological studies should soon determine whether cal-

cineurin is the relevant target of these immunosuppressants in vivo and is thus a key molecule in signal transmission pathways emanating from both the TCR in T lymphocytes and the IgE receptor in mast cells.

Experimental Procedures

Materials

Fresh calf thymus, brain, and spleen were obtained from Research 87 (Revere, MA). Bovine brain calcineurin and calmodulin were purchased from Sigma Chemical (St. Louis, MO). $^{45}CaCl_2$ was purchased from New England Nuclear (Cambridge, MA). ^{32}P-labeled phosphorylated peptide substrate, bovine brain calcineurin, and rabbit anti-calcineurin IgG were generous gifts from Dr. Claude B. Klee (Department of Biochemistry, National Cancer Institute). Goat anti-rabbit IgG conjugated with alkaline phosphatase and the alkaline phosphatase substrates BCIP and NBT were obtained from Pierce (Rockford, IL). Glutathione–Sepharose 4B was from Pharmacia LKB (Uppsala, Sweden). 506BD was prepared by Thomas J. Wandless and Patricia K. Somers in the Harvard laboratory.

Preparation of Fusion Proteins GCyP and GFK

The construction of a glutathione S-transferase–cyclophilin C fusion gene and purification of GCyP were achieved as described (Friedman and Weissman, 1991). To construct the GFK fusion gene, FKBP12 was amplified by the polymerase chain reaction from an FKBP12 coding plasmid (pRFS) using two primers: 5′ primer, 5′-CAGGACACAGGATCCATGGGC GTGCAGGTGGA-3′; 3′ primer, 5′-GCTGGCTAACGAATTCAAGGGAGGAGGCCATTCCTGTCAT-3′. The amplified fragment was purified by phenol–chloroform extraction and ethanol precipitation. It was then digested with EcoRI and BamHI and cloned into pGEX-2T, which had been linearized with the same restriction enzymes. The fusion construct pGFK12 was transformed into E. coli XA90, in which the expression of GFK can be induced with IPTG.

To purify GFK, a 1 liter LB culture of XA90/pGFK12 was incubated at 37°C. At an OD_{600} of 0.65, the culture was induced with 1 mM IPTG. The cells were harvested 6 hr after induction, resuspended in 20 ml of 20 mM Tris–HCl (pH 7.8) containing 1 mM PMSF, and lysed by two passes through a French press at 12,000 psi. The nucleic acids were precipitated by addition of 0.2 vol of neutralized 2% protamine sulfate solution to the crude lysate followed by centrifugation (20,000 × g, 20 min). The crude cell extract was then fractionated with ammonium sulfate, and the 40%–80% protein pellet was resuspended in 30 ml of 20 mM Tris–HCl (pH 7.8) and dialyzed against 4 liters of the same buffer.

The dialyzed protein solution was first purified with glutathione–Sepharose as described previously (Smith and Johnson, 1988). The glutathione (5 mM) eluate from the glutathione–Sepharose affinity column was directly loaded onto a DE-52 column that had been equilibrated with 5 column vols of 20 mM Tris–HCl (pH 7.8). The column was then washed with another 5 column vols of the buffer, and the fusion protein was eluted with a step gradient of 0–200 mM KCl. GFK-containing fractions were collected and used directly.

Preparation of Tissue Extracts of Bovine Brain, Thymus, and Spleen

Fresh bovine brain, thymus, and spleen were homogenized (1:3, wt/vol) in 20 mM Tris–HCl (pH 6.8), 0.25 mM NaCl, 2 mM β-mercaptoethanol, 0.02% NaN_3, 1 mM PMSF, and 5% glycerol. The homogenate was centrifuged at 8,000 × g for 4 hr. The supernatant was separated, and the pellet was resuspended in an equal volume of the same buffer. Centrifugation at 8,000 × g for 4 hr gave a second supernatant. The two supernatants were mixed (1:1, vol/vol) and centrifuged at 30,000 × g for 45 min. The supernatant was then filtered through a 0.45 μm filter and kept at 4°C before use.

Adsorption of Calcineurin–Calmodulin with GFK–FK506 and GCyP–CsA Using Glutathione–Sepharose

The crude tissue extracts were preincubated with glutathione–Sepharose (about 1:100 to 1:200 dilution of the Sepharose) at 4°C for 1–3 hr to remove the endogenous glutathione-binding proteins, including glutathione S-transferases. A typical incubation mixture has a total volume of 0.2 ml, consisting of buffer A (50 mM Tris–HCl [pH 7.5], 100 mM NaCl, 2 mM $MgCl_2$, 2 mM $CaCl_2$), 2 μM GFK (or GCyP), 20 μM FK506 (or rapamycin or CsA), and 0.05 to 0.1 ml of tissue extract. After incubation at 4°C on a Nutator for 1.5 hr, 25 μl of 50% (vol/vol) glutathione–Sepharose in buffer A was added, and incubation was continued for 0.5 to 2 hr. The Sepharose beads were precipitated by centrifugation in a microcentrifuge at 8,000 × g for 2 min. The glutathione–Sepharose beads were washed three times with 0.5 ml of buffer A containing 0.2% Triton X-100. The washed glutathione–Sepharose was then resuspended in 25 μl of 2 × SDS sample buffer, heated in boiling water for 3 min, and centrifuged for 2 min. The supernatant was subjected to SDS–PAGE followed by silver staining. For purification of the target proteins from calf thymus and brain extracts, each of the components was scaled up proportionally and the proteins were eluted with 20 mM EGTA in 50 mM Tris–HCl (pH 7.4), 1 mM dithiothreitol after three washes with buffer A containing 0.2% Triton X-100.

Western Blot Analysis of Calcineurin, Gel Mobility Shift of Calmodulin and Calcineurin B, and Detection of Calcineurin B by ^{45}Ca Autoradiography

For the Western blot analysis of calcineurin A and B, the proteins were subjected to 12% SDS–PAGE followed by electroblotting onto nitrocellulose using the Bio-Rad Mini-blotting apparatus. Development of the blot with rabbit anti-calcineurin IgG and alkaline phosphatase–conjugated goat anti-rabbit IgG was performed as previously described (Burnette, 1981). To detect the calcium-dependent gel mobility shift of calmodulin and calcineurin B, Ca^{2+} (1 mM) or EGTA (5 mM) was added to the protein solution in SDS sample loading buffer before loading the gel. The $^{45}Ca^{2+}$ binding to calcineurin B and calmodulin and subsequent autoradiography were carried out as previously described (Maruyama et al., 1984).

Phosphatase Assay of Calcineurin

The assay was carried out as described by Manalan and Klee (1983). The substrate used, provided by C. B. Klee, was a synthetic peptide corresponding to the phosphorylation site of the RII subunit of cAMP-dependent protein kinase (DLDVPIPGRFDRRVSVAAE), which was phosphorylated with ^{32}P-labeled ATP at the serine residue. The assay buffer consists of 40 mM Tris–HCl (pH 7.5), 0.1 M NaCl, 6 mM $MgCl_2$, 0.1 mM $CaCl_2$, 0.1 mg/ml bovine serum albumin, and 0.5 mM dithiothreitol. The assay mixture (60 μl) contained 40 nM calcineurin, 60 nM calmodulin (when present), and 2 μM phosphopeptide in addition to the assay buffer. It was found that the presence of methanol (3%) inhibits the phosphatase activity significantly. Therefore, the drug solutions were prepared in DMSO as follows. DMSO stock solutions of the drugs were prepared (3000× final concentration) and then diluted 100× with the assay buffer; 2 μl was added to the incubation mixture, giving a final concentration of DMSO of less than 0.04% in the final assay mixture. The incubations were carried out at 30°C for 10 min before the reaction was stopped by addition of the stop solution (5% trichloroacetic acid, 0.1 M potassium phosphate) and loaded onto 0.5 ml Dowex AG 50W-X8 (200–800 mesh; Bio-Rad) columns. After the $^{32}P_i$ was eluted from the column, it was mixed with 12 ml of ScintiVerse II (Fisher Scientific) and counted on a Beckman LS1801 Liquid Scintillation System.

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A mammalian protein targeted by G1-arresting rapamycin–receptor complex

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THE structurally related natural products rapamycin and FK506 bind to the same intracellular receptor, FKBP12, yet the resulting complexes interfere with distinct signalling pathways[1,2]. FKBP12–rapamycin inhibits progression through the G1 phase of the cell cycle in osteosarcoma[3], liver[4,5] and T cells[6,7] as well as in yeast[8], and interferes with mitogenic signalling pathways that are involved in G1 progression[9,10], namely with activation of the protein p70[S6k] (refs 5, 11–13) and cyclin-dependent kinases[11,14–16]. Here we isolate a mammalian FKBP–rapamycin-associated protein (FRAP) whose binding to structural variants of rapamycin complexed to FKBP12 correlates with the ability of these ligands to inhibit cell-cycle progression. Peptide sequences from purified bovine FRAP were used to isolate a human cDNA clone that is highly related to the DRR1/TOR1 and DRR2/TOR2 gene products from *Saccharomyces cerevisiae*[8,17,18]. Although it has not been previously demonstrated that either of the DRR/TOR gene products can bind the FKBP–rapamycin complex directly[17,19], these yeast genes have been genetically linked to a rapamycin-sensitive pathway and are thought to encode lipid kinases[17–20].

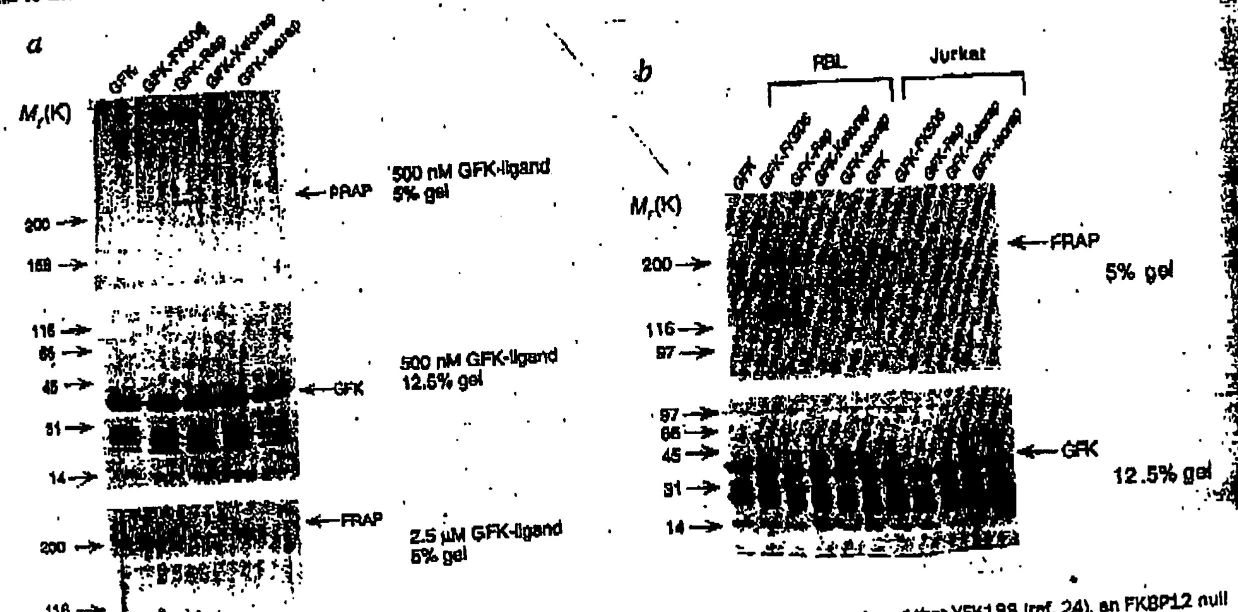
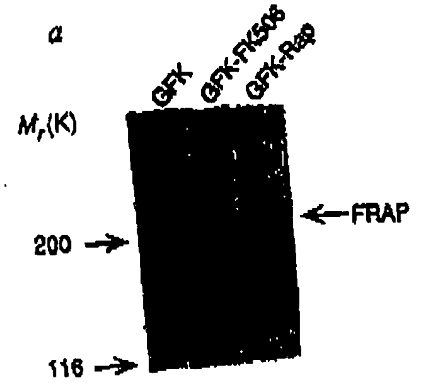


FIG. 1 Identification of FRAP protein in three mammalian cell lines. a, GFK alone or individual GFK–ligand complexes were added to MG-63 cell lysates (2×10^7 cells per condition) to a final concentration of either 500 nM or 2.5 μM and the mixtures incubated for 10 min at 4 °C. Fusion protein complexes were recovered by glutathione-affinity chromatography and the proteins detected by silver staining after 5% SDS–PAGE. Because of compression, FRAP is not resolved by 12.5% SDS–PAGE, so both 5% and 12.5% gels are shown. The amount of FRAP that was retained by affinity chromatography saturated at concentrations of GFK–Rap greater than 500 nM in these experiments and in others using concentrations of GFK–Rap ranging from 100 nM to 5 μM (data not shown). b, GFK alone or individual GFK–ligand complexes were added to a final concentration of 500 nM to lysates prepared from either 2×10^8 Jurkat T lymphocytes or 10^8 rat basophilic leukaemia (RBL) cells per condition. Lysates were treated as in a. FKBP12, but not FKBP13 or FKBP25 (ref. 23) is able to mediate the actions of rapamycin in *S. cerevisiae*. In addition, we found that YFK188 (ref. 24), an FKBP12 null strain, could be complemented with GFK (P. K. Martin, B. Gladstone, G. Weiss, D. T. Hung, S.L.S., in preparation). Thus the GST appendage of the fusion protein does not preclude binding of the biologically relevant target to the GFK–rapamycin complex in yeast.

METHODS. MG-63, Jurkat and RBL cells were grown in media containing 10% FBS and lysed at 4 °C in PINT buffer (150 mM NaCl, 50 mM Tris-HCl, pH 7.5, 2 mM EDTA, 2 mM EGTA, 25 mM NaF, 100 μM Na_3VO_4, 25 mM 2-glycerophosphate, 0.2 mM PMSF, 1 μg ml^{-1} leupeptin, 1 μg ml^{-1} pepstatin A and 2 mM DTT) containing 0.5% Triton X-100. Lysates were clarified by centrifugation at 25,000*g*, and the Triton X-100 in the supernatant was diluted to 0.33% by adding 0.5 vol PINT buffer. GFK prebound to stoichiometric quantities of FK506, keto- iso- or unmodified rapamycin was added to lysates as described. Each condition was then passed through a 250-μl glutathione-Sepharose column, which was washed with PINT buffer containing 0.5 M NaCl and 0.3% Triton X-100.



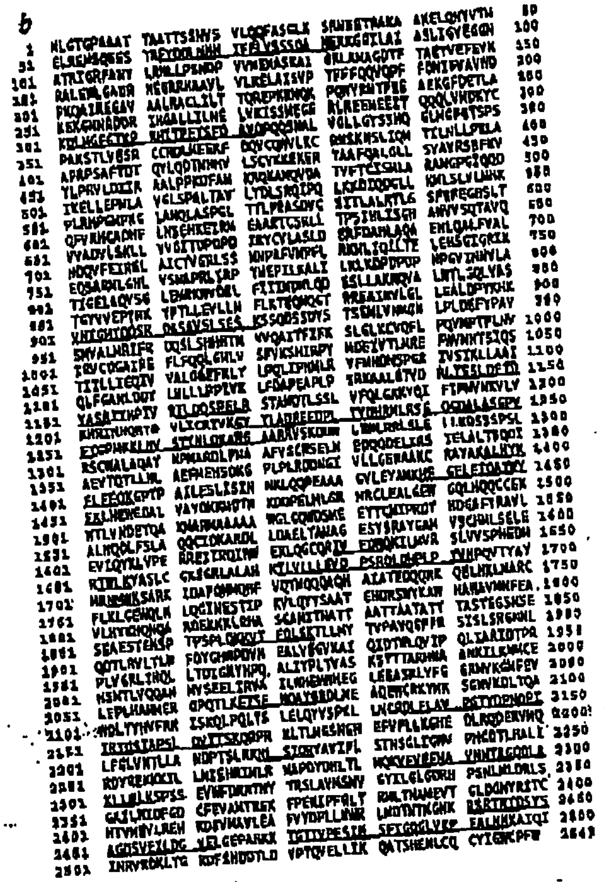
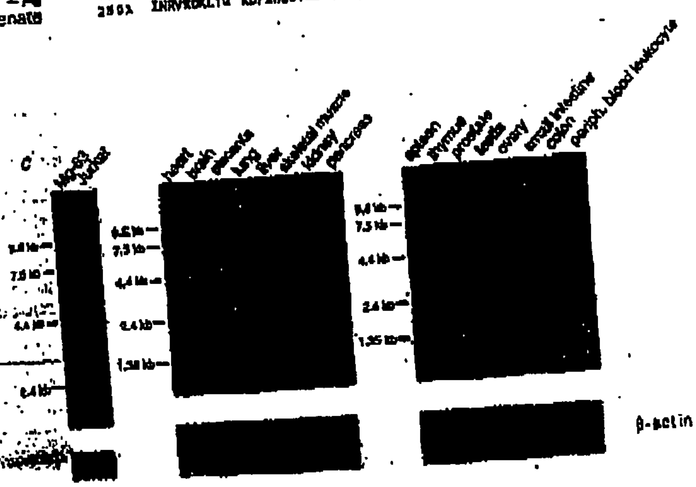


FIG. 2 Purification of FRAP from bovine brain and cDNA cloning of human FRAP. a, Fivefold-enriched bovine FRAP (S-column eluate; see below) was conditioned with (100 nM) glutathione-S-transferase-FKBP12 fusion protein (GFK), GFK-FK506 or GFK-Rap. Complexes with fusion proteins were recovered by glutathione-affinity chromatography and detected as described in Fig 1 legend. We also found FRAP in bovine liver and thymus. b, Predicted translational product of the human FRAP cDNA clone. Bovine FRAP peptide sequences aligned to human FRAP are indicated by underlined segments. In the reading frame shown translational stop codons were not encountered upstream of the initiating methionine. c, Northern blot analysis of human tissue, Jurkat T cell and MG-63 cell poly $(A)^+$ RNA. The Jurkat/MG-63 and multiple tissue Northern blots (Clontech) were hybridized with ^{32}P-labelled probes derived from the 182 bp PCR fragment and the 6.5-kb clone (text), respectively. Hybridization to human β-actin probe is shown as an internal control for loading.

METHODS. Bovine FRAP was purified by grinding 900 g of bovine brain in blender with 1 litre of PIP (0.3% Triton X-100, 50 mM sodium phosphate, pH 7.2, 2 mM EDTA, 2 mM EGTA, 25 mM NaF, 100 μM Na_3VO_4, 25 mM 2-glycerophosphate, 1 mM PMSF, 1 μg ml^{-1} leupeptin, 1 μg ml^{-1} pepstatin A, 1 mM benzamidine and 2 mM DTT). The homogenate was centrifuged at 25,000g and the supernatant (20 g total protein) was loaded onto a 1 litre S-Sepharose (Pharmacia) column. The column was then washed with PIP and eluted with PINP (PIP with 1M NaCl). GFK–rapamycin was added to the pooled eluate to a final concentration of 100 nM and recovered by glutathione-affinity chromatography. FRAP was resolved by SDS–PAGE and transferred to PVDF. Following digestion with trypsin or endoproteinase Lys-C (Boehringer Mannheim) bFRAP peptides were microsequenced[28]. The Jurkat T cell cDNA library (Stratagene) was constructed through random and oligo dT priming of cytoplasmic oligo-dT purified RNA (ref. 25). cDNA screening, Jurkat and MG-63 RNA isolation and northern blotting and were performed by procedures similar to those previously described[26]. A 182 bp fragment was amplified from a human brain stem library (Stratagene) and labelled by incorporation of ^{32}P-dCTP in the course of reamplification by PCR. The sequences were analysed using BLAST (ref. 26) and the University of Wisconsin GCG (ref. 27) software. The human FRAP cDNA sequence has been submitted to Genbank.



We used two structural variants of rapamycin, 16-keto-rapamycin (S. D. Meyer and S.L.S., manuscript in preparation) and 25,26-iso-rapamycin[21], to identify any biologically relevant targets of the FKBP-rapamycin complex. Both variants bind tightly to human FKBP12, as shown by their ability to inhibit rotamase activity of the recombinant protein (K_i values were 0.2 nM for rapamycin[4], 2 nM for keto-rapamycin, and 0.1 nM for iso-rapamycin). But the variants are about two orders of magnitude less potent than rapamycin in preventing the progression through G1 of MG-63 human osteosarcoma cells. The values of IC_{50} (half-maximal inhibitory concentration) estimated from dose–response curves are 0.1 nM, 7.5 nM and 50 nM for rapamycin, keto- and iso-rapamycin, respectively. Thus the complexes of iso- and keto-rapamycin with FKBP12 should bind to the FKBP12-rapamycin target less effectively than FKBP12-rapamycin itself.

A fusion protein of glutathione-S-transferase with FKBP12 (GFK) was used to identify candidates for the biologically relevant targets of FKBP12–rapamycin. MG-63 cells were lysed by detergent and complexes of GFK–rapamycin, GFK–FK506 or GFK alone were added individually to clarified lysate at a final concentration of 500 nM or 2.5 μM (Fig. 1a). A protein of approximate relative molecular mass 220,000 (M_r ~220K) was detected in the GFK–rapamycin sample by SDS-PAGE and silver staining (Fig. 1a, lane 3). This FKBP-rapamycin-associated protein (FRAP) was not retained with GFK–FK506 or GFK alone (Fig. 1a, lanes 1 and 2). No other rapamycin-specific proteins were detected by silver staining (Fig. 1a) or by a similar

affinity purification procedure using lysates from [^{35}S] methionine-labelled cells (data not shown). The GFK–ketorapamycin and GFK–isorapamycin complexes bound FRAP less effectively than GFK–rapamycin; at concentrations of 500 nM, the keto- and iso- complexes were unable to retain the 220K protein (Fig. 1*a*, lanes 4, 5), whereas at higher concentrations of the complexes (2.5 μM) detectable quantities of FRAP were retained (Fig. 1*a*, lanes 4, 5). This is consistent with the finding that these compounds are still strong cell-cycle inhibitors, albeit less potent than rapamycin itself. Thus, the binding of GFK–ligand complexes to FRAP correlates with the ability of the ligands to impede G1 progression in MG-63 cells. FRAP was also detected in Jurkat T-lymphocyte cells and rat basophilic leukaemia cells (Fig. 1*b*), two mammalian cell lines that are also sensitive to rapamycin[6,22]. No other rapamycin-specific bands were observed in each case.

FRAP purified from bovine brain (bFRAP) had a similar specificity for GFK-ligand (Fig. 2*a*). Microsequencing of bFRAP proteolytic fragments (298 amino acids in total, Fig. 2*b*) led to the design of a pair of degenerate oligonucleotides for use in the polymerase chain reaction (PCR). A 182 bp PCR product allowed for the isolation of overlapping clones from a human Jurkat T cell λZAP II cDNA library, yielding 7.6 kb of contiguous sequence. Using these cDNA sequences as probes, a band migrating at approximately 8.5 kilobases was detected by Northern blot analysis of oligo dT purified RNA isolated from a variety of human tissues and cell lines (Fig. 2*c*). The human cDNA sequence encodes an amino-acid open reading frame (ORF) and aligns with 99% identity to the bFRAP peptides (Fig. 2*b*). As N-terminal peptide sequence from purified bovine FRAP was not obtained, the initiating methionine shown in Fig. 2*b* is unconfirmed. The predicted molecular mass of this ORF (~300K) is greater than that inferred by the mobility of FRAP during SDS-PAGE (above).

Human FRAP is highly related to the *DRR1/TOR1* and *DRR2/TOR2* gene products. Overall it is 44% identical to DRR1/TOR1 and 46% identical to DRR2/TOR2. The region of greatest homology to DRR1/TOR1 and DRR2/TOR2 lies in the C-terminal 660 amino acids of human FRAP (57% and 59% identical, respectively). In addition, this region has homology to several known phosphatidylinositol kinases (21% identity on average), including mammalian phosphatidylinositol 3-kinase[17,18] (PI3K), a yeast PI3K *VPS34* (refs 17 and 18) and *PIK1* (ref. 20). These similarities indicate that FRAP may also have phosphatidylinositol kinase activity.

Through the introduction of minute structural changes in rapamycin, this study implicates FRAP as a mediator of G1 cell cycle progression in mammalian cells. Identification of FRAP as the target of FKBP12-rapamycin together with the earlier demonstration of calcineurin as the target of FKBP12-FK506 (ref. 2) addresses a fascinating aspect of immunophilin research, namely that the immunophilin FKBP12 can bind two distinct natural products and thereby gain the ability to bind two distinct signalling molecules involved in cell cycle entry and progression. Further biochemical characterization of this unique mammalian protein should elucidate its role in propagating the mitogen-initiated signals that lead to the activation of p70[S6k] and cyclin-Cdk complexes. □

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Functional dissection of the yeast Cyc8–Tup1 transcriptional co-repressor complex

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DNA-BINDING repressor proteins mediate regulation of yeast genes by cell type (Mcm1/α2 and a1/α2), glucose (Mig1) and oxygen (Rox1) (refs 1–4 respectively). An unusual feature of all these regulatory pathways is that transcriptional repression requires two physically associated proteins[5] that do not bind DNA Cyc8(Ssn6) and Tup1. The Cyc8–Tup1 complex has been proposed to be a co-repressor that is recruited to target promoters by pathway-specific DNA-binding proteins[6], but the specific functions of the individual proteins are unknown. Here we show that when it is bound upstream of a functional promoter through the LexA DNA-binding domain, Tup1 represses transcription in the absence of Cyc8. Deletion analysis indicates that Tup1 contains at least two non-overlapping transcriptional repression regions with minimal primary sequence similarity, and a separable Cyc8-interaction domain. These Tup1 domains, which do not include the β-transducin motifs[7], are necessary and partially sufficient for Tup1 function. We suggest that Tup1 performs the repression function of the Cyc8–Tup1 co-repressor complex, and that Cyc8 serves as a link with the pathway-specific DNA-binding proteins.

It has been previously shown that Cyc8 can repress transcription in a Tup1-dependent manner when bound upstream of the intact *CYC1* promoter through the heterologous LexA DNA-binding domain[6]. Similarly, a LexA–Tup1 hybrid protein confers a 16-fold reduction of expression from a promoter containing four LexA operators upstream of the *CYC1* promoter (Table 1). LexA–Tup1 and LexA–Cyc8 also repress expression of a *his3* gene containing a single LexA operator upstream of the Tc TATA element (Fig. 1*a*), suggesting that they can inhibit basal transcription. Surprisingly, LexA–Tup1 retains almost its entire

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Isolation of a Protein Target of the FKBP12-Rapamycin Complex in Mammalian Cells*

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The immunosuppressive drug, rapamycin, interferes with an undefined signaling pathway required for the progression of G_1-phase T-cells into S phase. Genetic analyses in yeast indicate that binding of rapamycin to its intracellular receptor, FKBP12, generates a toxic complex that inhibits cell growth in G_1 phase. These analyses implicated two related proteins, TOR1 and TOR2, as targets of the FKBP12-rapamycin complex in yeast. In this study, we have used a glutathione S-transferase (GST)-FKBP12-rapamycin affinity matrix to isolate putative mammalian targets of rapamycin (mTOR) from tissue extracts. In the presence of rapamycin, immobilized GST-FKBP12 specifically precipitates similar high molecular mass proteins from both rat brain and murine T-lymphoma cell extracts. Binding experiments performed with rapamycin-sensitive and -resistant mutant clones derived from the YAC-1 T-lymphoma cell line demonstrate that the GST-FKBP12-rapamycin complex recovers significantly lower amounts of the candidate mTOR from rapamycin-resistant cell lines. The latter results suggest that mTOR is a relevant target of rapamycin in these cells. Finally, we report the isolation of a full-length mTOR cDNA that encodes a direct ligand for the FKBP12-rapamycin complex. The deduced amino acid sequence of mTOR displays 42 and 45% identity to those of yeast TOR1 and TOR2, respectively. These results strongly suggest that the FKBP12-rapamycin complex interacts with homologous ligands in yeast and mammalian cells and that the loss of mTOR function is directly related to the inhibitory effect of rapamycin on G_1- to S-phase progression in T-lymphocytes and other sensitive cell types.

The structurally related macrolides, rapamycin and FK506, are potent immunosuppressants that inhibit signal transduction pathways required for T-cell activation and growth (for review, see Ref. 1). FK506 interferes with a Ca^{2+}-dependent signaling event that couples T-cell antigen receptor occupancy to transcription of the genes encoding interleukin-2 (IL-2)[1] and several other cytokines. In contrast, rapamycin inhibits IL-2-stimulated T-cell proliferation by blocking cell cycle progression from late G_1 into S phase (2, 3).

FK506 and rapamycin bind to a family of intracellular receptors termed FK506 binding proteins (FKBPs). The most well characterized member of the FKBP family is a 12-kDa isoform, FKBP12 (4–6). Studies in yeast and mammalian cells have established a novel mechanism of action for rapamycin and FK506. According to this model, the binding of rapamycin or FK506 to FKBP12 generates a toxic complex that interferes with a specific component of the intracellular signaling machinery. For example, the FKBP12·FK506 complex binds to and inhibits the Ca^{2+}-calmodulin-regulated protein serine-threonine phosphatase, calcineurin (7, 8), which catalyzes an event necessary for IL-2 gene transcription (9, 10). In contrast, the FKBP12-rapamycin complex does not interact with calcineurin, and the molecular target(s) of this complex in lymphoid cells remains undefined.

Rapamycin's inhibitory action on G_1-phase progression in yeast (11) parallels its anti-proliferative effect on IL-2-stimulated T-lymphocytes. Genetic studies in yeast may therefore provide clues regarding the mechanism of action of rapamycin in mammalian cells. As predicted from the gain-of-function model described above, disruption of the yeast *FKB1* locus, which encodes FKBP12, yields viable cells that are resistant to rapamycin (11). A screen for additional rapamycin-resistant yeast mutants led to the discovery of two novel genes, *TOR1* and *TOR2* (12, 13). The *TOR1* and *TOR2* genes encode related proteins containing putative lipid kinase domains homologous to those found in both yeast and mammalian phosphatidylinositol 3-kinases. Whether the FKBP12-rapamycin complex binds targets in mammalian cells that are homologous to the yeast TOR proteins remains an important but unsettled question.

In this study, we have isolated from mammalian tissue extracts a high molecular mass protein that binds specifically to the FKBP12-rapamycin complex. The amino acid sequence of this protein, which we have designated a mammalian target of rapamycin (mTOR), suggests that it represents a mammalian homolog of the yeast TOR proteins. Furthermore, comparisons of rapamycin-sensitive and -resistant mutant subclones of a murine T-lymphoma cell line indicate that drug resistance is highly correlated with reduced binding of mTOR to the FKBP12-rapamycin complex. These studies strongly suggest that mTOR represents a relevant target of the FKBP12-rapamycin complex in T-lymphocytes.

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§ The first two authors made equivalent contributions to this work.

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[1] The abbreviations used are: IL-2, interleukin-2; FKBPs, FK506 binding proteins; ORF, open reading frame; mTOR, mammalian target of rapamycin; GST, glutathione S-transferase; GSH, glutathione; PBS, phosphate-buffered saline; PAGE, polyacrylamide gel electrophoresis; p70S6K, p70 S6 kinase; PCR, polymerase chain reaction.

MATERIALS AND METHODS

Cell Lines—YAC-1 T-lymphoma cell lines were maintained in 10 mM Hepes-buffered RPMI 1640 medium (pH 7.2) supplemented with 10% fetal bovine serum, 2 mM L-glutamine, and 50 μM 2-mercaptoethanol. The derivation of rapamycin-resistant and -sensitive YAC-1 subclones has been described previously (14).

Production of Glutathione S-Transferase (GST)-FKBP12 Fusion Protein—A cDNA encoding the open reading frame of human FKBP12 was cloned into the *Bam*HI site of pGEX2T (Pharmacia). The recombinant plasmid was transformed into *E. coli* strain JM101. To produce the GST-FKBP12 fusion protein, one-liter cultures of transformed bacteria were grown at 37 °C with shaking to an optical density (A_{600}) of 0.5. The culture was induced with 0.5 mM isopropyl-1-thio-β-D-galactopyranoside, and the incubation was continued for an additional 3 h. The bacteria were harvested and lysed by sonication according to the protocol supplied with the pGEX2T vector. GST-FKBP12 was purified from the cleared bacterial extract by chromatography over a glutathione (GSH)-agarose column. The column was washed with phosphate-buffered saline (PBS) containing 1% Triton X-100 (PBST), and the fusion protein was eluted with PBST containing 30 mM GSH. Peak fractions were pooled and dialyzed into PBS at 4 °C. The purified GST-FKBP12 displayed FK506 binding and peptidyl-prolyl isomerase activities equivalent to those observed with native FKBP12.

Preparation of Tissue Extracts—Frozen rat brains were obtained from Harlan Sprague-Dawley (Indianapolis, IN) and were homogenized with a Polytron (Brinkmann Instruments, Westbury, NY) in two volumes of 50 mM Tris-HCl, 100 mM NaCl, 15 mM β-glycerophosphate, 1 mM CaCl$_2$, 1 mM MgCl$_2$ (pH 7.4), containing 10% glycerol, 2 mM 2-mercaptoethanol, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml leupeptin, 5 μg/ml pepstatin A, and 5 μg/ml aprotinin. The homogenate was centrifuged for 60 min at 11,500 × *g*, and the resulting supernatant was recentrifuged for 1 h at 140,000 × *g*. Proteins in the supernatant were precipitated by dropwise addition of saturated ammonium sulfate solution to a final concentration of 30% (v/v). Precipitated proteins were collected by centrifugation and were resolubilized in 2 ml of homogenization buffer/brain equivalent.

YAC-1 cells (2 × 10^8 cells/sample) were harvested from culture medium, washed 2 times in PBS, and resuspended in hypotonic buffer (10 mM Tris-HCl, 5 mM MgCl$_2$ (pH 7.4)) containing 50 μM digitonin and the protease inhibitor mixture described above. After 20 min on ice, the cells were lysed in an all-glass Dounce homogenizer. The hypotonic buffer was subsequently converted to brain homogenization buffer by the addition of the appropriate amounts of the solutes described above. All remaining steps in the preparation of T-cell extracts were identical to those described above.

Affinity Purification of Drug FKBP12 Binding Proteins—To prepare the affinity matrix, GST-FKBP12 (100 μg) was immobilized on 20 μl of GSH-coupled agarose beads. The beads were washed 4 times with homogenization buffer containing 0.02% Nonidet P-40. Brain or YAC-1 cell extracts (0.4–4 mg of protein/ml) were precleared with GSH-agarose loaded with GST-FKBP12. The cleared extracts were rotated for 1 h with immobilized GST-FKBP12 in the absence or presence of 10 μM rapamycin or FK506. The precipitates were washed 4 times with homogenization buffer, and bound proteins were eluted with 2 × Laemmli sample buffer (15). Proteins were resolved by SDS-polyacrylamide gel electrophoresis (PAGE) and detected by silver staining (Bio-Rad).

Preparative scale purification of the FKBP12-rapamycin-binding protein was carried out for amino acid sequence analysis. GST-FKBP12 was immobilized on GSH-agarose and incubated with brain extract (3–5 mg of protein) in the presence of 10 μM rapamycin as described above. After the batch adsorption step, the remaining supernatant was discarded, and a fresh aliquot of brain extract was added to the same tube. The serial adsorption process was repeated until approximately 20 mg of extract protein had been incubated with each aliquot of GST-FKBP12-coupled beads. The bound proteins were eluted and separated by SDS-PAGE as described above.

Amino Acid Sequencing—After SDS-PAGE, the proteins were fixed and stained in 20% methanol, 0.5% glacial acetic acid containing 0.2% (w/v) Coomassie Blue. Based on the staining intensities of the target protein-containing gel fragments relative to that of a known standard (rabbit skeletal muscle myosin), we estimate that approximately 8 μg of purified protein was submitted to the Mayo Peptide Synthesis and Protein Sequencing Facility for further analysis. The appropriate regions of the gel were excised, and the protein was subjected to in-gel digestion with modified sequencing grade trypsin (Boehringer-Mannheim) (16). The tryptic peptides were injected onto 2.1 × 220-mm Aquapore 300 high performance liquid chromatography column (Applied Biosystems) and were eluted from the column at a solvent flow rate of 0.2 ml/min. The initial eluting solvent was 1% solvent B (40% acetonitrile in 0.1% trifluoroacetic acid) and 99% solvent A (5% acetonitrile in 0.1% trifluoracetic acid). After 8 min, a linear gradient to 10% solvent B was performed over 5 min. At 13 min, a second linear gradient to 100% solvent B was imposed over the next 67 min. The optical density of the eluate was monitored at 215 nm, and 0.1-ml fractions were collected. Amino acid microsequencing was performed on an Applied Biosystems Model 475A protein sequencer.

Isolation of a cDNA Clone Encoding a Mammalian TOR Homolog—Amino acid microsequencing yielded an unambiguous LELAVPG sequence that was also present in the yeast TOR proteins but not in any other known protein. To isolate the full-length cDNA encoding mTOR, primers based upon the LELAVPG sequence and upon regions of conservation between the yeast TOR proteins and the phosphatidylinositol 3-kinase catalytic subunit were used to generate a PCR fragment that could be used to probe a cDNA library. The first set of four sense primers were degenerate 17-mers covering the amino acid sequence ELAVPG obtained from the purified protein. The primers were GAR YTN GCN GTN CCT GG, GAR YTN GCN GTN CCC GG, GAR YTN GCN GTN CCA GG, and GAR YTN GCN GTN CCG GG. The first set of three antisense primers covered the conserved amino acid sequence (I/F)HIDFG, from the yeast TOR proteins (12, 13). The primers were: NCC RAA RTC (A/G/T)AT RTG AA, NCC RAA RTC (A/G/T)AT RTG GA, and NCC RAA RTC (A/G/T)AT RTG TA. The primers were used in PCR reactions in all 12 possible combinations with rat brain cDNA as the template and with Ultima *Taq* Polymerase (Perkin-Elmer) as the polymerase. The PCR conditions were 95 °C for 30 s, 56 °C for 30 s, and 72 °C for 30 s.

The products of the first reaction were used as templates in a second PCR reaction to generate a nested product. The second set of four degenerate sense primers corresponded to the amino acid sequence (D/E)D(I/L)RQD (12, 13). The primers were GAN GAY ATN CGN CAA GA, GAN GAY ATN CGN CAG GA, GAN GAY ATN AGN CAA GA, and GAN GAY ATN AGN CAG GA. The second set of four degenerate antisense primers covered the conserved sequence DRH(P/N)SN (12, 13). The four primers were (A/G)TT N(G/C) (A/T) N(G/T) (G/T) (A/G)TG NCG GTC, (A/G)TT N(G/C) (A/T) N(G/T) (G/T) (A/G)TG NCG ATC, (A/G)TT N(G/C) (A/T) N(G/T) (G/T) (A/G)TG NCT ATC, and (A/G)TT N(G/C) (A/T) N(G/T)(G/T) (A/G)TG NCT GTC. Sixteen independent PCR reactions were performed using 0.5 μl of each of the 12 initial PCR reaction products as templates (192 reactions total). Eleven reactions yielded products of the predicted size (about 450 base pairs), and these fragments were subcloned into pUC19 and sequenced.

Of the eleven PCR products sequenced, one yielded a deduced amino acid sequence homologous to the yeast TOR proteins. That PCR product was labeled by random priming (Prime-It, Stratagene) with hexamers in the presence of [α-^{32}P]dATP and was used to screen 1.2 million recombinant phage from an oligo(dT) and randomly primed rat brain cDNA library (Stratagene). Prehybridization, high stringency hybridization, and washing conditions were performed according to the manufacturer's specifications. Positive phage were plaque-purified, and the *Eco*RI cDNA inserts were excised from DNA purified from plate lysates of the cloned phage. The *Eco*RI cDNA inserts were subcloned into pUC19 and sequenced with either Sequenase or *Taq* polymerase on an Applied Biosystems 373A automated DNA sequencer. Each strand was sequenced a minimum of 4 times each.

A 7.1-kilobase pair partial cDNA was isolated from the first screen. After sequencing, *Eco*RI-linked primers were synthesized and used in a PCR reaction to isolate the first 450 base pairs in the 7.1-kilobase pair partial cDNA. The sense primer was GAA TTC ATG AAY CCN GCN TTT GT, and the antisense primer was GAA TTC GTN GKR TAR WAY TCG TC. The product was digested with *Eco*RI and subcloned into pUC19. The product was excised from *Eco*RI, labeled as described above, and used to rescreen the same library. Several clones were isolated that overlapped with the 5′ end of the 7.1-kilobase pair clone by as much as 4000 nucleotides and extended the sequence upstream by as much as 1600 nucleotides.

Because the open reading frame (ORF) still extended to the 5′ end of the contiguous sequence, the remainder of the cDNA was isolated by RACE (rapid amplification of cDNA ends). The template was 5′ Race-Ready rat brain cDNA (Clontech). The 5′ anchor primer was supplied by the manufacturer. The antisense primers corresponding to the rat mTOR gene were GAA TTC CCA CCT TCC ACT CCA ATG for the primary reaction and GAA TTC CAT ATG CTT GGG ACA GCC CCT GCC ACG in the secondary PCR reaction. Sequencing of the RACE products demonstrated that we had obtained the remainder of the ORF.

In Vitro Transcription and Translation of mTOR cDNA—The 7.1-

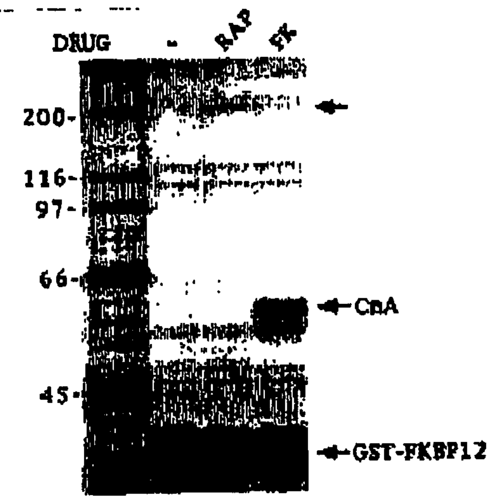


FIG. 1. **Binding of rat brain-derived proteins to GST-FKBP12-drug complexes.** Rat brain extracts (1 mg of protein/sample) were incubated with 100 µg of GST-FKBP12 coupled to GSH-agarose beads. Precipitations were performed in the absence of drug (−) or in the presence of 10 µM rapamycin (*RAP*) or 10 µM FK506 (*FK*). Precipitated proteins were eluted, resolved by SDS-PAGE through a 8.75% gel, and visualized by silver staining. *Lane 1* shows the molecular mass calibration standards, with actual masses (kDa) designated on the *left*. Unlabeled *arrow* on the *right* indicates the GST-FKBP12-rapamycin-specific binding protein. Labeled *arrows* on *right* indicate the locations of the 57- and 61-kDa isoforms of the calcineurin A (*CnA*) subunit and the GST-FKBP12 fusion protein.

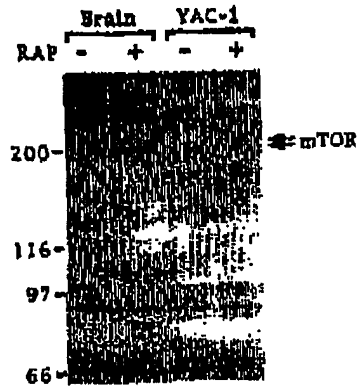


FIG. 2. **Isolation of mTOR from YAC-1 T-lymphoma cells.** Extracts were prepared from rat brain and YAC-1 T-lymphoma cells, and equal amounts of total protein (0.45 mg/sample) were precipitated with GSH-agarose-coupled GST-FKBP12 in the absence (−) or presence (+) of 10 µM rapamycin (*RAP*). Bound proteins were separated by SDS-PAGE in a 7.5% gel and were visualized by silver staining as described in the Fig. 1 legend. *Double arrow* on the *right* indicates the location of brain and YAC-1 cell-derived mTOR.

kilobase pair mTOR cDNA clone was excised from pUC19 by digestion with *Eco*RI, and the gel-purified insert was cloned into the *Eco*RI site of pSP72 (Promega). A recombinant pSP72 plasmid containing the 7.1-kilobase pair cDNA in the proper orientation relative to the T7 RNA polymerase promoter site was identified by restriction mapping and [illegible]. The cDNA was transcribed and translated in the presence of [^{35}S]methionine using the TnT T7 coupled reticulocyte lysate system (Promega). One-tenth volume (5 µl) of the translation reaction was denatured in Laemmli sample buffer and subjected to SDS-PAGE. The remainder of the translation reaction was diluted with homogenization buffer containing 1 mg/ml bovine serum albumin. The diluted sample was divided into aliquots and incubated with GSH-agarose-bound fusion proteins in the absence or presence of rapamycin or FK506, and protein-binding assays were performed as described above. The bound proteins were separated by SDS-PAGE. The gels were treated with EN^{3}HANCE (DuPont NEN), and the ^{35}S-labeled translation products were detected by fluorography at −70 °C.

RESULTS

Detection of Drug-FKBP12 Binding Proteins in Tissue Extracts—Our strategy for the detection of rapamycin-FKBP12 ligands was based on the affinity purification technique previously employed for the identification of calcineurin as an intracellular target for the FK506·FKBP12 complex (7). Tissue extracts were incubated with GSH-agarose-coupled GST-FKBP12 in the absence or presence of rapamycin or FK506, and bound proteins were analyzed by SDS-PAGE. As shown in Fig. 1, rapamycin-loaded GST-FKBP12 precipitated a high molecular mass (>200 kDa) protein from rat brain extracts. The interaction of this protein with GST-FKBP12 was specifically dependent on the presence of rapamycin, as the protein was not found in precipitates prepared without drug or with FK506. As predicted, the GST-FKBP12·FK506 complex recovered relatively abundant 57- and 61-kDa proteins corresponding to known isoforms of the calcineurin A subunit (7). Both the calcineurin B subunit (18 kDa) and calmodulin (17 kDa) were also evident when the latter precipitates were electrophoresed through higher percentage acrylamide gels (data not shown).

Expression of Drug-FKBP12 Binding Proteins in T-lymphoma Cells—The results described above prompted additional studies to determine whether similar FKBP12-rapamycin binding proteins were expressed in T-lymphocytes. Earlier reports documented that rapamycin inhibits both the proliferation and the cytokine responsiveness of the murine T-lymphoma cell line, YAC-1 (17–19), indicating that YAC-1 cells express a functionally important target of rapamycin. The results in Fig. 2 depict a comparison of the proteins recovered from rat brain and YAC-1 cell extracts by the GST-FKBP12-rapamycin complex. In the presence of rapamycin, the immobilized GST-FKBP12 specifically precipitated proteins with similar electrophoretic mobilities from both YAC-1 cell and rat brain extracts. Henceforth, these high molecular mass binding proteins will be provisionally designated as mTOR. Interestingly, the GST-FKBP12-rapamycin precipitates from YAC-1 cells contained two high molecular mass proteins, with the lower band co-migrating with mTOR isolated from rat brain. The recovery of the higher molecular mass species from different YAC-1 cell extracts appeared to correlate with that of the lower band (see Fig. 3), suggesting that these are either post-translationally modified versions of the same protein or that the upper band represents a distinct protein whose interaction with the FKBP12-rapamycin complex is contingent on the binding of mTOR.

Recovery of Putative Targets of Rapamycin from Mutant T-lymphoma Sublines—Recent studies characterized a panel of rapamycin-resistant and -sensitive clones derived from the YAC-1 T-lymphoma cell line (14). The mutant clones were generated by treatment of YAC-1 cells with ethylmethanesulfonate, followed by limiting dilution cloning in rapamycin-containing culture medium. Clonal populations were selected for resistance to the antiproliferative effect of rapamycin. Further characterization of these clones revealed that several (R19, 4R16, and 10R13) displayed a stable, drug-resistant phenotype, whereas others (R103, R125) were fully sensitive to rapamycin in short term cultures. The three rapamycin-resistant clones expressed normal amounts of FKBP12 and retained wild-type levels of sensitivity to FK506, suggesting that the resistant phenotype was due to an alteration located downstream of FKBP12 (14). By analogy to the previously described yeast *TOR* mutants (11–13), we reasoned that one or more of

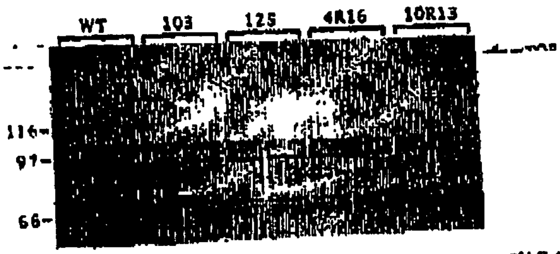


FIG. 3. **Recovery of mTOR from wild-type and mutant YAC-1 clones.** *Left panel* shows equivalent amounts of extract (0.45 mg of protein/sample) from wild-type (WT) YAC-1 cells, and the mutant clones R103 (103), R125 (125), 4R16, and 10R13 were precipitated with GST-FKBP12 in the absence (−) or presence (+) of 10 μM rapamycin (RAP). Bound proteins were separated and visualized as described in the legend to Fig. 2. *Double arrow on right* indicates the location of mTOR. *Right panel* shows extracts that were prepared from rat brain, wild-type (WT) YAC-1 cells, a rapamycin-sensitive revertant subclone derived from R19 (R19rev), and the original, rapamycin-resistant R19 clone (R19of). Recovery of mTOR was determined as described above.

the rapamycin-resistant YAC-1 clones might express mutant mTOR with altered binding to the GST-FKBP12-rapamycin complex.

The silver-stained gel shown in Fig. 3, *left panel*, compares the recoveries of mTOR from wild-type (WT) YAC-1 cells and clones. The immobilized GST-FKBP12-rapamycin complexes precipitated mTOR from both of the rapamycin-sensitive clones, R103 and R125, at levels comparable with those obtained from wild-type cells. In contrast, substantially lower levels of mTOR were consistently precipitated from the rapamycin-resistant clones 4R16 and 10R13. These results indicated that the reduced recovery of mTOR by the GST-FKBP12-rapamycin complex was correlated with the rapamycin-resistant phenotype in the mutant YAC-1 clones (see "Discussion").

The correlation between decreased binding of mTOR to the FKBP12-rapamycin complex and resistance to rapamycin was further substantiated by studies performed with a spontaneous revertant of the rapamycin-resistant R19 clone. After over 6 months in culture medium without rapamycin, a stock culture of R19 cells was found to express an increased level of sensitivity to rapamycin. A drug-sensitive revertant clone, designated $R19_{rev}$, was subsequently isolated from this variant population. Binding experiments revealed that the GST-FKBP12-rapamycin complex consistently precipitated a higher amount of mTOR from $R19_{rev}$ cells than from parental, rapamycin-resistant R19 cells (Fig. 3, *right panel*). Thus, the reacquisition of rapamycin sensitivity in the $R19_{rev}$ clone was accompanied [illegible] FKBP12-rapamycin complex in the rapamycin-resistant YAC-1 mutants strongly suggested that mTOR is a relevant target of [illegible] genes had been cloned genetically from rapamycin-resistant yeast strains by their ability to confer rapamycin resistance to wild-type strains. The high molecular masses of both the yeast *TOR* gene products and purified mTOR suggested that mTOR might be a mammalian counterpart to yeast TOR1 and TOR2. We therefore hypothesized that mTOR might be related to the proteins encoded by yeast *TOR1* and *TOR2*. To confirm this, the cDNA encoding rat mTOR was isolated from a rat brain cDNA library.

In order to clone the cDNA, mTOR was purified from rat brain as described previously and digested with trypsin, and the resulting tryptic peptides were subjected to amino acid sequencing. One of the peptides yielded an unambiguous heptapeptide sequence, LELAVPG. This heptapeptide is 100% identical to an amino acid sequence found in both the yeast TOR1 and TOR2 proteins (12, 13). In contrast, the heptapeptide [illegible] nucleic acid (GenBank release 89) data bases. In particular, the LELAVPG sequence is not contained within any of the regions of homology between the yeast TOR proteins and the mammalian phosphatidylinositol 3-kinase catalytic subunits (p110). We designed a cloning strategy based in part on the mTOR- and yeast TOR-specific LELAVPG sequence. We further postulated that those regions in the yeast TOR proteins that share homology with p110 would also be conserved in mTOR. A set of "sense" PCR primers was designed that corresponded to the LELAVPG sequence. The use of these primers insured that cDNAs encoding mammalian phosphatidylinositol 3-kinase catalytic subunits would not be amplified because the encoded heptapeptide is not present in any phosphatidylinositol 3-kinase nor is it present in any other known protein. A set of "antisense" PCR primers was designed that corresponded to the amino acid sequence (I/F)HIDFG, which is well conserved between the yeast TOR proteins and p110. These sets of primers were used in PCR reactions (see "Materials and Methods" for details) with rat brain cDNA as the template. A small amount of the product from this primary PCR reaction was used in a secondary PCR reaction. The "sense" primers in the secondary reaction corresponded to the sequence ED(I/L)RQD, which is conserved between the yeast TOR proteins and the [illegible] ment of the size predicted assuming that mTOR is homologous to the yeast TOR1 and TOR2 proteins. [illegible] sequencing revealed that it encoded a protein fragment with 70% identity to regions in TOR1 and TOR2 that correspond to their putative lipid kinase domains. In contrast, alignment of the mTOR fragment with the catalytic domain of bovine p110 demonstrated a lower level (32.5%) of amino acid identity. Therefore, we concluded that the PCR product we had isolated encoded a mammalian homolog of the yeast TOR proteins rather than a phosphatidylinositol 3-kinase catalytic subunit or related protein.

The PCR product was used to screen a randomly-primed rat brain cDNA library (see "Materials and Methods"), and a 7.1-

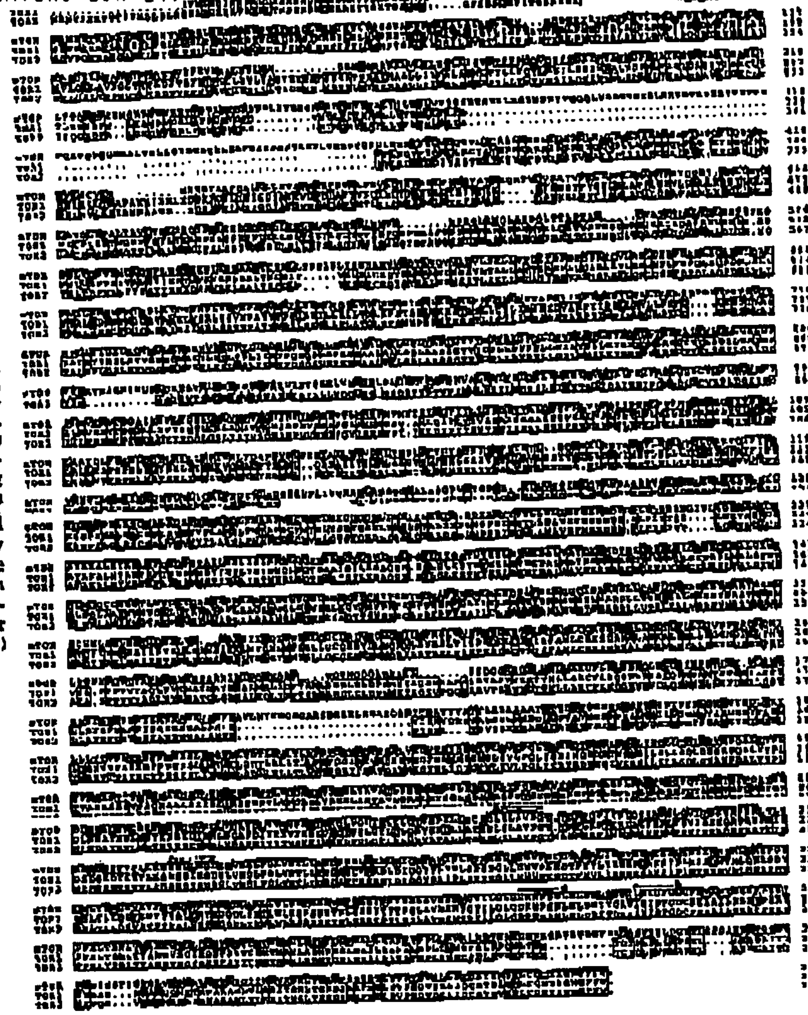


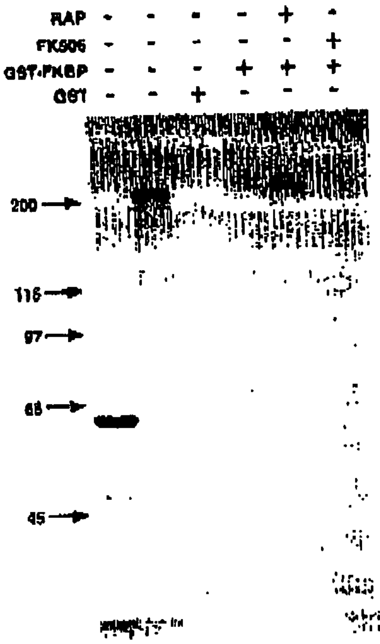
FIG. 4. Alignment of the translation product of the mTOR ORF with the [illegible] TOR genes. Identical residues are *boxed*. Amino acid number is indicated at the *right* of each line. The LELAVPG sequence obtained from peptide sequencing is indicated by the *heavy overline*. The PCR reaction (*a* and *b*) and the second PCR reaction (*c* and *d*) are indicated by *arrows*. The partial cDNA used to probe the cDNA libraries is contained between the *c* and *d* primers. Alignments were performed using GCG (Genetics Computer Group, University of Wisconsin, Madison) software (27).

kilobase pair clone was isolated. Sequencing of the 7.1-kilobase pair clone revealed that it encoded a protein highly homologous to the yeast TOR proteins. Moreover, the encoded protein contained the LELAVPG sequence obtained by protein sequencing. The ORF extended to the 5' end of the cDNA indicating that it might not be complete. This was confirmed by the results of Northern analysis of rat brain mRNA with the 7.1-kilobase pair clone as a probe. The Northern suggested that the full-length mRNA might be as large as 9.5 kilobase pairs.[2] A PCR product derived from the extreme 5' end of the 7.1-kilobase pair clone was used to rescreen the library. Three additional cDNAs were isolated that overlapped with the 7.1-kilo[illegible] The ORF [illegible] remainder of the cDNA was isolated by RACE. The complete cDNA contains 8554 nucleotides and encodes a protein of 2549 amino acids (Fig. 4) with a predicted molecular mass of 289 kDa. The presence of an in-frame stop that it is probably the initiator methionine and that a full-length cDNA clone has been isolated.

The translated ORF is shown aligned with the yeast TOR1 and TOR2 proteins in Fig. 4. The deduced amino acid sequence of mTOR is 42.4% and 44.9% identical to TOR1 and TOR2, respectively. The location of the LELAVPG heptapeptide (amino acids 2136–2142), which was obtained by protein sequencing and was used to design the first "sense" PCR primer (the *a* primer) is shown *overlined*. The deduced amino acid sequence of mTOR also contains the conserved sequences from yeast TOR proteins and the p110 subunit of mammalian phosphatidylinositol 3-kinase that were used to design the other three [illegible] 2339–2351), and LHIDFG (residues 2357–2362). The [illegible] both yeast TOR proteins in all of these regions except for the conservative leucine for isoleucine substitutions in the EDL-RQD and LHIDFG peptides. The original PCR product that was isolated encodes the region located between the *a* and *d* [illegible]

In summary, there are at least three features about the clone that confirm that we have isolated the cDNA encoding rat mTOR. First, the LELAVPG sequence obtained by peptide

[2] M. Martin and G. Wiederrecht, unpublished results.



derived translation products. A partial cDNA clone encoding mTOR residues 468-1259 (see Fig. 4) was transcribed and translated *in vitro* in the presence of [^{35}S]methionine (see "Materials and Methods"). Five µl [illegible] translation products [illegible] primed with a control luciferase cDNA are shown in *lane 1*. In *lanes 2-6*, the mTOR translation products were precipitated with the indicated GST-fusion proteins, in the absence or presence of FK506 or rapamycin (*RAP*). Bound polypeptides were solubilized and separated by SDS-PAGE. ^{35}S-labeled polypeptides were detected by fluorography.

that of the protein that we have purified. Third, mTOR is highly similar in sequence to both of the yeast TOR proteins. Searches of two protein data bases (Swiss-Prot release 28 and NBRF-PIR release 40) and a translated nucleic acid data base (GenBank release 83) with the mTOR amino acid sequence confirmed that mTOR is more highly homologous to the yeast TOR proteins than it is to any other known proteins.

mTOR cDNA Encodes a Ligand for the FKBP12-Rapamycin Complex—To determine whether the isolated mTOR cDNA encodes a FKBP12-rapamycin-binding protein, the 7.1-kilobase pair cDNA clone described above was transcribed and translated in the presence of [^{35}S]methionine. The 5' terminal [illegible] consensus context (20) for the initiation of translation. The full-length translation product encoded by the 7.1-kilobase pair [illegible] idues 468-2549 (Fig. 4). Direct electrophoretic analysis of the mTOR cDNA-primed translation mixture revealed a closely spaced doublet of ^{35}S-labeled polypeptides with apparent molecular masses of greater than 200 kDa (Fig. 5, *lane 2*). Incubation of the translation mixture with immobilized GST only or GST-FKBP12 resulted in the recovery of barely detectable amounts of these radiolabeled polypeptides [illegible] binding of the mTOR-derived polypeptides to GST-FKBP12 was not enhanced by the addition of FK506 to the precipitation reaction (*lane 5*). In contrast, rapamycin dramatically increased the binding of the ^{35}S-labeled translation products to the GST-FKBP12-coupled beads (*lane 6*). These results demonstrate that the isolated mTOR cDNA encodes a protein that binds directly and specifically to the FKBP12-rapamycin complex.

DISCUSSION

In this study, we used an affinity purification technique to isolate a direct ligand of the FKBP12-rapamycin complex from rat brain and murine T-lymphoma cells. Amino acid sequencing of the rat brain-derived target protein, designated mTOR, led to the isolation of a full-length cDNA whose deduced amino acid sequence displayed a high degree of identity to the yeast *TOR1* and *TOR2* gene products. TOR1 and TOR2 are highly related proteins that perform both overlapping and distinct functions in the regulation of the yeast cell cycle (11). The present data do not allow us to determine whether the mTOR cDNA encodes a functional homolog of *TOR1* or *TOR2*. Nevertheless, the present findings lend strong support to the hypothesis that the FKBP12-rapamycin complex interferes with G_1-phase progression in yeast and mammalian cells by acting on a highly homologous set of target proteins.

Nonallelic noncomplementation among recessive, rapamycin-resistant alleles of *FPR1*, *TOR1*, and *TOR2* in yeast provided the first genetic evidence that these gene products interacted in the presence of rapamycin (11). The biochemical [illegible] FKBP12-rapamycin complex interacts with a mammalian TOR homolog but also demonstrate for the first time that a TOR [illegible] lin-drug complex. [illegible] model that proposes that the FKBP12-rapamycin complex exerts its growth-inhibitory and cytotoxic effects in yeast by inhibiting the functions of TOR1 and TOR2 (21, 22). Our studies suggest that this model can be extended to mammalian T-lymphocyte [illegible] phoma cell line. Earlier results had suggested that the resistant phenotypes of the 4R18, 10R13, and R19 clones were due to mutations located downstream of FKBP12 (14). In this study, we demonstrate that GST-FKBP12-rapamycin complexes precipitate significantly lower amounts of mTOR from rapamycin-resistant YAC-1 clones than from their sensitive counterparts. Northern analyses have shown that the sensitive and resistant cell lines express equivalent levels of mTOR mRNA, indicating that the observed differences in mTOR recovery are not due to alterations in mTOR mRNA turnover.[2] These results support the hypothesis that the resistant clones [illegible] essential catalytic function of mTOR intact. T-cells expressing such a mutation would not experience a loss of mTOR function [illegible] transit G_1 phase at normal rates. A mutation leading to a reduction in the binding affinity of mTOR for the FKBP12-rapamycin complex, with otherwise normal mTOR catalytic activity, would be predicted to act in a dominant fashion in

[2] R. T. Abraham, unpublished observations.

heterozygotes. Consistent with this prediction, heterokaryon fusions between rapamycin-sensitive, parental YAC-1 cells and rapamycin-resistant 4R16, 10R13, or R19 cells displayed mutant-type resistance to rapamycin.[4]

Previous studies showed that the resistance of the 4R16, 10R13, and R19 clones to rapamycin was not absolute. In short term cultures, the proliferation of these cells was inhibited by 20–40% in the presence of micromolar concentrations of rapamycin (14). The residual sensitivities of the 4R16, 10R13, and R19 clones to rapamycin suggests that these cells express mutations that reduce, but do not abrogate, the ability of the FKBP12-rapamycin complex to interact with mTOR. Sequencing of mTOR cDNAs from rapamycin-sensitive and -resistant YAC-1 clones might begin to localize the binding site for the FKBP12-rapamycin complex. However, resistance-conferring mutations may not be restricted to the mTOR gene locus. A recent report suggests that the lipid kinase domains of yeast TOR1 and TOR2 contain homologous serine residues that may require phosphorylation to interact with the rapamycin-FKBP12 complex (22). Expression of a mutant kinase with decreased phosphotransferase activity toward mTOR might explain the observed decrease in mTOR recovery from the rapamycin-resistant YAC-1 clones.

The function of the TOR family members in cellular regulation remains enigmatic. The C-terminal 600 amino acid residues of mTOR are approximately 65% identical to TOR1 and TOR2. This region bears significant homology to the lipid ki-[illegible] experiments indicate that the putative lipid kinase domains of yeast TOR1 and TOR2 play essential but interchangeable roles in cell cycle regulation (22). Nonetheless, biochemical proof that the TOR proteins possess phosphoinositide kinase activity [illegible] malian p110 is actually a dual specificity phosphotransferase [illegible] 3-kinase activity, p110 catalyzes the [illegible] ulatory serine residue in the p85 subunit of the phosphatidylinositol 3-kinase heterodimer. It is conceivable that the TOR family members also function as dual specificity, lipid-protein kinases in yeast and mammalian cells.

The inhibitory effect of rapamycin on G_1-phase progression in yeast parallels its antiproliferative action on IL-2-stimulated T-cells. Given the apparent conservation of TOR catalytic function in yeast and mammalian cells, it is tempting to speculate that the TOR proteins function as "universal" regulators of G_1-phase progression in eukaryotic cells. However, studies with rapamycin as a pharmacologic probe suggest that TOR function is rate limiting for passage through G_1-phase in some, but not all, mammalian cell types. Rapamycin strongly inhibits the growth of most hematopoietic cell lines, but exerts marginal to negligible growth-inhibitory effects on many mesenchymal or epithelial cell lines (24).[3] A possible explanation is that rapamycin-insensitive cell types compensate for the loss of [illegible] complex.

An alternative hypothesis is that mTOR plays a permissive rather than an obligate role in G_1 to S phase progression in mammalian cells. The sensitivities of different cell types to rapamycin might therefore reflect intrinsic variations in the dependence of the rate of G_1-phase progression on mTOR function. Notably, exposure of cycling T-cells to rapamycin appears to slow, rather than prohibit, the entry of late G_1-phase cells into S phase.[5] In yeast, a double disruption of *TOR1* and *TOR2* results in overt growth-arrest in G_1 phase, whereas disruption of *TOR1* only yields a "slow-growth phenotype" (22). The growth-inhibitory effect of rapamycin on lymphoid and other sensitive mammalian cell types might resemble the phenotype of the yeast *TOR1* mutant more closely than that of doubly-disrupted *TOR1/TOR2* mutant.

The relatively tissue-specific effect of rapamycin on proliferation contrasts sharply with the ability of this drug to completely block p70 ribosomal S6-kinase (p70S6K) activation in most, if not all, mammalian cell types (24–26). Indeed, earlier studies of mutant YAC-1 clones demonstrated that resistance to the anti-proliferative effect of rapamycin segregated with resistance to the inhibitory effect of this drug on p70S6K activity (14). Thus, the protein kinase/phosphatase cascade that regulates p70S6K activity appears universally dependent on an upstream function performed by rapamycin-sensitive mTOR(s). Whether inhibition of p70S6K activity is related to the growth-inhibitory effect of rapamycin remains unclear. Perhaps mTOR functions as an upstream regulator of additional signal transduction pathways that are unrelated to cell cycle control. The expression of high levels of mTOR in adult brain, a tissue with a relatively low proliferative potential, supports this notion. Clearly, further biochemical characterization of this protein will be required to elucidate its roles in triggering G_1-phase progression and other cellular responses.

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Addendum—During the review of this manuscript, Brown *et al.* (28) and Sabatini *et al.* (29) reported the isolation of human and rat cDNA clones encoding FKBP12-rapamycin target proteins termed FRAP and [illegible] of FRAP and RAFT1 with that of mTOR indicates that all three cDNAs

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[5] R. T. Abraham and F. J. Dumont, unpublished observations.

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(54) Title: MONOCLONAL ANTIBODIES TO CYCLOSPORINGS

(57) Abstract

Novel monoclonal antibodies capable of distinguishing between cyclosporins, e.g. Cyclosporine, and metabolites, e.g. Cyclosporins 17 and 18, are produced, e.g. starting from novel cyclosporins having an activated coupling group, e.g. activated carboxy group, e.g. (i) [(O-succinimidooxysuccinyl)-Thr]^2-Cyclosporine and (ii) [(N-ε-succinimidooxysuccinyl)-(D)Lys]^8-Cyclosporine. Cyclosporin starting materials required for the production of cyclosporins of type (ii), e.g. [(D)Lys]^8-Cyclosporine are also new and additionally have utility in the preparation of novel labelled cyclosporin derivatives, as well as antibodies and antisera generally. Also claimed are novel antigenic conjugates and hybridoma cell lines used in the production of antibodies and antisera as aforesaid as well as assay kits comprising novel antisera, antibodies and/or labelled cyclosporins as aforesaid.

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CG	Congo	LI	Liechtenstein	SN	Senegal
CH	Switzerland	LK	Sri Lanka	SU	Soviet Union
CM	Cameroon	LU	Luxembourg	TD	Chad
DE	Germany, Federal Republic of	MC	Monaco	TG	Togo
DK	Denmark	MG	Madagascar	US	United States of America
FI	Finland	ML	Mali		
FR	France				

MONOCLONAL ANTIBODIES TO CYCLOSPORINS

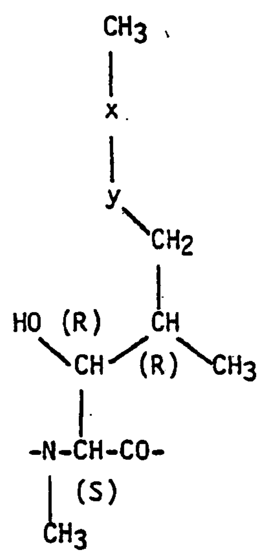
The present invention relates to monoclonal antibodies to cyclosporins, in particular to monoclonal antibodies capable of distinguishing between cyclosporins and metabolites thereof and suitable for use in diagnostic/assay kits, as well as to novel hybridoma cell lines used in the production of said monoclonal antibodies and diagnostic/assay kits comprising said monoclonal antibodies. In addition the invention relates to novel cyclosporins and immunogenic conjugates comprising them, used for the generation of monoclonal antibodies as aforesaid and also useful for the generation of regular polyclonal antisera suitable for diagnostic/assay kit use, as well as to the product antisera and antibodies and diagnostic/assay kits comprising them. The invention also relates to labelled derivatives of said novel cyclosporins, themselves suitable for diagnostic/assay kit use, as well as to diagnostic/assay kits comprising them.

The cyclosporins comprise a class of structurally distinctive, cyclic, poly-N-methylated undecapeptides commonly possessing pharmacological, in particular immunosuppressive, anti-inflammatory and anti-parasitic activity. The first of the cyclosporins

to be isolated was the naturally occurring fungal metabolite Cyclosporine, also known as cyclosporin A, of formula A

MeBmt-αAbu-Sar-MeLeu-Val-MeLeu-Ala-(D)Ala-MeLeu-MeLeu-MeVal
1 2 3 4 5 6 7 8 9 10 11 (A)

wherein -MeBmt- represents the N-methyl-(4R)-4-but-2E-en-1-yl-4-methyl-(L)threonyl residue of formula B



(B)

in which -x-y- is -CH=CH- (trans).

Since the original discovery of Cyclosporine, a wide variety of naturally occurring cyclosporins have been isolated and identified and many further non-natural cyclosporins have been prepared by total- or semi-synthetic means or by the application of modified culture techniques. The class comprised by the cyclosporins is thus now substantial and includes for example the naturally

occurring cyclosporins A through Z [c.f. Kobel et al. European Journal of applied Microbiology and Biotechnology 14, 237 - 240 (1982) and poster presented by Traber et al., 24th. Interscience Conference on Antimicrobial Agents and Chemotherapy, Washington, October 8 - 10, (1984)]; as well as various non-natural or artificial cyclosporins, including dihydro-cyclosporins (in which the group -x-y- of the -MeBmt- residue (see formula B above) is saturated, e.g. as disclosed in the US Patents Nos. 4,108,985; 4,210,581 and 4,220,641, cyclosporins in which the -MeBmt- residue is present in isomeric or N-desmethyl form [c.f. European patent no. 0 034 567 and "Cyclosporin A", Proc. Internat. Conference on Cyclosporin A, Cambridge (U.K.) September 1981, Ed. D.J.G. White, Elsevier Press (1982) - both describing the total-synthetic method for the production of cyclosporins developed by R. Wenger] and cyclosporins in which incorporation of variant amino acids at specific positions within the peptide sequence is effected. Examples of such cyclosporins as disclosed in the above art references include e.g. $[Thr]^2$-, $[Val]^2$-, $[Nva]^2$- and $[Nva]^2$-$[Nva]^5$-Cyclosporine (also known as cyclosporins C, D, G and M respectively) and dihydro-$[Val]^2$-Cyclosporine (also known as dihydrocyclosporin D).

[In accordance with now conventional nomenclature for the cyclosporins, these are defined throughout the present specification and claims by reference to the structure of Cyclosporine (i.e. cyclosporin A). This is done by first indicating those residues in the molecule which differ from those present in Cyclosporine and then applying the term "Cyclosporine" to characterise the remaining residues which are identical to those present in Cyclosporine. At the same time the prefix "dihydro" is employed to designate cyclosporins wherein the -MeBmt- residue is hydrogenated (-dihydro-MeBmt-), i.e. wherein -x-y- in formula B is

$-CH_2-CH_2-$. Thus [Thr]^2-Cyclosporine is the cyclosporin having the sequence shown in formula A, but in which -αAbu- at the 2-position is replaced by -Thr-, and dihydro-[Val]^2-Cyclosporine is the cyclosporin having the sequence shown in formula A but in which -MeBmt- at position 1 is hydrogenated and -αAbu- at the 2 position is replaced by -Val-.

In addition, amino acid residues referred to by abbreviation, e.g. -Ala-, -MeVal- etc... are, in accordance with conventional practice, to be understood as having the (L)-configuration unless otherwise indicated. Residue abbreviations preceded by "Me", as in the case of -MeLeu- represent N-methylated residues. The individual residues of the cyclosporin molecule are numbered, as in the art, clockwise and starting with the residue -MeBmt- (or -dihydro-MeBmt-) in position 1. The same numerical sequence is employed throughout the present specification and claims.]

Because of their unique immunosuppressive activity, the cyclosporins have attracted very considerable attention not only in medical and academic circles, but also in the lay press. Cyclosporine itself is now commercially available and commonly employed to prevent rejection following allogenic organ, e.g. heart, heart-lung, kidney and bone-marrow transplant, as well as more recently in the treatment of various auto-immune and related diseases and conditions. Both dihydro-[Val]^2-Cyclosporin and [Nva]^2-Cyclosporin are under extensive clinical investigation as potential successors to Cyclosporine.

Dosaging of cyclosporins, e.g. Cyclosporine, however presents particular difficulties. Since metabolic conversion rates tend to be patient specific and the therapeutic range narrow, effective dosaging is highly subject specific and requires the establish-

ment of appropriate individual serum levels. Regular monitoring of cyclosporin plasma concentrations is thus an essential prerequisit for effective treatment. To this end a number of high pressure liquid chromatography (HPLC), radioimmunoasssay (RIA) and fluoroimmunoassay (FIA) systems have been developed. However, HPLC methods, whilst highly specific are difficult and cumbersome to use in practice and the current commercially available RIA system based on sheep polyclonal antiserum has met with criticism because of its lack of specificity. Development of cyclosporin, e.g. Cyclosporine, specific monoclonal antibodies capable of distinguishing between therapeutically administered cyclosporins and their metabolites in man has accordingly for a long time been an urgent practical as well as purely scientific goal, since these would have the advantage of offering the same potential specificity as HPLC methodology, whilst retaining the advantage of ease of application provided by conventional immunoassay systems. In addition the provision of such cyclosporin-specific monoclonal antibodies would provide a vital new research tool permitting e.g. the comparative investigation of cyclosporin conformation and definition of cyclosporin receptor requirements etc...

Since the original discovery of Cyclosporine, numerous attempts have been made to produce monoclonal antibodies reactive to cyclosporins. Since cyclosporins, e.g. Cyclosporine, themselves have little immunogenic activity, a common approach has been to proceed employing an immunogenic, e.g. hapten-protein, conjugate, e.g. derived by coupling of immunoglobulins via the hydroxy group available at -Thr^2- in [Thr]^2-Cyclosporine employing conventional coupling techniques, e.g. with EDCI [N-ethyl-N'-(3-dimethylamino-propyl)carbodiimide.2HCl] or MCDI [N-cyclohexyl-N'-[β-(N-methyl-morpholino)ethyl]-carbodiimide.p.toluene sulfonate] as coupling

agent. Attempts in this manner have however failed and where monoclonal antibodies have been obtained, these have been found to have relatively low specificity for Cyclosporine, or to be specific with respect to the carrier protein or the coupling reagent employed rather than Cyclosporine, or to be highly cross-reactive with the coupling agent. In no instance has it proved possible to produce monoclonal antibodies identifiable as distinguishing between e.g. Cyclosporine and metabolites thereof, e.g. the metabolites Cyclosporine 17 and Cyclosporine 18 hereinafter specifically described. In addition such attempts have led to the production of monoclonal antibodies to Cyclosporine of the type IgM only and hence in any event essentially inappropriate for use in any form of regular, e.g. clinical, assay kit. The production of monoclonal antibodies having specific reactivity with cyclosporins and capable of distinguishing between individual cyclosporins and their metabolites, e.g. between Cyclosporine and its metabolites in man, and suitable for use in an assay system has thus remained a major goal.

In accordance with the present invention it has now surprisingly been found that monoclonal antibodies reactive to cyclosporins and meeting the various objectives discussed above, in particular capable of distinguishing between cyclosporins and metabolites thereof, can be produced via essentially conventional immunisation/fusion/cloning techniques, employing immunogenic conjugates comprising a cyclosporin as hapten at the initial immunisation step, if the conjugate is prepared by coupling of the carrier to the cyclosporin by the agency of an activated coupling group, e.g. if conjugate synthesis is effected employing a cyclosporin having an activated coupling group as starting material. In particular using such immunogenic conjugates it is possible to obtain monoclonal antibodies capable of fine discrimination

between cyclosporins and metabolites thereof bearing even single variant groupings on individual residues, e.g. in the case of Cyclosporine, being reactive with Cyclosporine while exhibiting low cross-reactivity with, for example, its metabolites Cyclosporine 17 and/or Cyclosporine 18.

In addition to at last providing the means for development of convenient monoclonal assay systems, e.g. for use in clinic, the present invention also provides a means for the further purification of cyclosporin metabolites and, since it may be anticipated that monoclonal antibodies will be obtainable by application of the general methods of the invention, which may mimic receptor sites, the characterisation of potential endogenous cyclosporin-like molecules. The significance of the present invention from both a practical and a purely scientific stand point will be thus readily apparent.

As indicated above, the immunogenic conjugates required in the practice of the invention are prepared by direct coupling of a carrier, e.g. protein molecule, with a cyclosporin by the agency of an activated coupling group. This may be effected, either by reaction of a carrier bearing an activated coupling group with a cyclosporin bearing an appropriate co-reactive substituent, e.g. hydroxy or amino group, e.g. as in the case of $[Thr]^2$-Cyclosporine or $[(D)Lys]^8$-Cyclosporine hereinafter described, or by reaction of a carrier with a cyclosporin having an activated coupling group, e.g. cyclosporin in which one of the amino acid residues present in the cyclosporin molecule has a side chain at the α-carbon atom comprising or bearing an activated coupling group. The said conjugates thus comprise a cyclosporin, hapten moiety, directly linked to a carrier moiety, rather than via an intervening coupling agent residue, as in the case of immunogenic

conjugates comprising a cyclosporin as hapten previously employed in the art, e.g. for raising regular polyclonal antisera.

By the term "activated coupling group" as used herein and throughout the accompanying claims is to be understood any group capable of direct reaction with an appropriate, co-reactive grouping, e.g. amino, hydroxy, thio group or the like, so as to provide a co-valent linkage, without requirement for use of a coupling agent to enable, effect or promote reaction. Thus in the case of cyclosporins bearing an "activated coupling group" this will be any group capable of direct reaction with a carrier molecule, e.g. protein molecule, to provide a co-valently linked conjugate with said carrier molecule, without requirement for use of a coupling reagent to enable, effect or promote coupling or reaction with said carrier molecule.

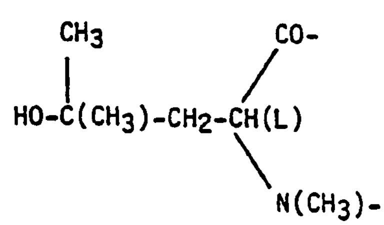
Groups suitable as activated coupling groups are well known in the art and include for example i) activated ester or activated carboxy groups, i.e. of formula -CO-OZ wherein Z is a carboxy activating group such as o- or p-nitrophenyl, 1-benztriazole, pentafluorophenyl or N-succinimido; ii) activated dithio groups, i.e. of formula -S-S-X wherein X is a dithio activating group such as 2-pyridyl; and iii) epoxy groups.

Suitable immunogenic conjugate carrier molecules, bearing an activated coupling group, e.g. epoxy group, as aforesaid, may be prepared in accordance with techniques known in the art, e.g. as described by Laumen et al., Tetrahedron Letters, 26 (4), 407 - 410 (1985). In accordance with the general methods of the present invention it is however preferred that the activated coupling group be provided on the cyclosporin which is to be coupled with the carrier, rather than vice versa.

In principle the activated coupling group may be present at any position around the cyclosporin molecule. In so far as transformations at the 1-position are of particular significance in cyclosporin metabolism, or in so far as major cyclosporin metabolites, e.g. in the case of Cyclosporine, Cyclosporine 17 and Cyclosporine 18, exhibit structural variation at the 1-position as described below, it is preferred that the activated coupling group be present at one or other of positions 2 to 11 inclusive, thus leaving the residue at the 1-position intact, preferably "unmasked" by the carrier, in the immunogenic conjugate subsequently obtained, and hence free to elicit specific antibody response. Generally it is appropriate if the activated coupling group is at the 2-position or at any of the positions 3, 5 to 8 or 10, especially 5 to 8 inclusive, whereby the 2- and 8-positions are particularly favoured.

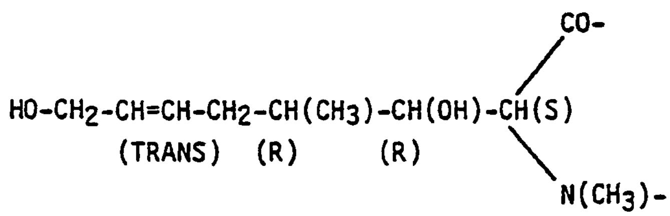
In the case of Cyclosporine major metabolic conversions occurring in man are:

I Terminal hydroxylation of $-MeLeu^{9}-$ to give the residue of formula E



(E);

II Terminal hydroxylation of -MeBmt[1]- to give the residue of formula F



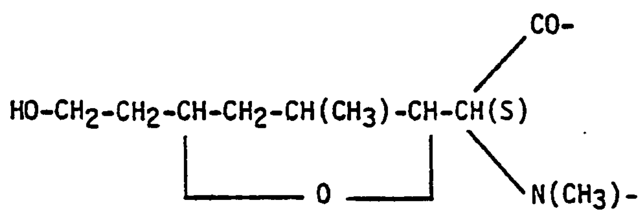
(F);

III Des-N-methylation of -MeLeu[4]- to give -Leu-;

IV Terminal hydroxylation of -MeLeu[4]- to give the residue of formula E above;

V Terminal hydroxylation of -MeLeu[6]- to give the residue of formula E above;

VI Terminal hydroxylation and ring-closure in -MeBmt[1]- to give the residue of formula G



(G)

Thus known metabolites of Cyclosporine (identified as Cyclosporine <u>1</u>, Cyclosporine <u>8</u> etc...) exhibit the following metabolic variations.

Cyclosporine 1 : I. Cyclosporine 8 : I + II. Cyclosporine 9 : I + III + V. Cyclosporine 10 : I + IV. Cyclosporine 16 : I + V. Cyclosporine 17 : II. Cyclosporine 18 : VI. Cyclosporine 21 : III.

[See G. Maurer et al, "Drug Metab. Disposit" 12, 120 - 126 (1984)].

Accordingly, for the preparation of monoclonal antibodies capable of distinguishing between Cyclosporine and metabolites thereof in man, it will be appropriate that the activated coupling group in the cyclosporin employed for immunogenic conjugate formation, be situated in a position other than the 1-, 4-, 6- or 9-position, and, in so far as Cyclosporine 17 and 18 represent major metabolites, at least in a position other than the 1-position. Thus again in the particular case of Cyclosporine, the 2- and 8-position are especially favoured.

Cyclosporins having an activated coupling group as described above may be prepared e.g. either:

i) by activation of an appropriate pre-existing precursor group (i.e. coupling group in non-activated form), e.g. conversion of the carboxy group of a cyclosporin having a carboxy-substituted α-amino acid residue (i.e. α-amino acid residue having a side chain at the α-carbon atom comprising or bearing a carboxy group), e.g. at the 2- or 8-position, into an activated carboxy group, by reaction with a carboxy activating agent; or

ii) by acylation or etherification of a cyclosporin having an amino- or hydroxy-substituted α-amino acid residue (i.e. α-amino acid residue having a side chain at the α-carbon atom comprising or bearing a hydroxy or amino group), e.g. hydroxy-substituted α-amino acid residue at the 2-position or amino- or hydroxy-substituted α-amino acid residue at the 8-position, with an acylating or alkylating agent bearing an activated coupling group.

Process step i) above may be carried out in accordance with standard techniques known in the art, e.g. for the activation of carboxy groups by reaction with a regular carboxy activating agent such as o- or p-nitrophenol, 1-hydroxy-benztriazole, pentafluorophenol or N-hydroxy-succinimide. Reaction is suitably carried out in the presence of a condensing agent such as EDCI.

Process step ii) may also be carried out in accordance with essentially conventional techniques. Thus amino or hydroxy groups may be suitably acylated by reaction with a derivative of a carboxylic acid in which the carboxy group is activated and which additionally bears an activated coupling group which is non-reactive with amino or hydroxy as the case may be, for example N-[(2-pyridyl)dithio-propion-1-yl]-succinimide, [the (2-pyridyl)-dithio moiety providing the activated coupling group (non reactive, in this instance, with both amino and hydroxy groups) and the -COO-succinimido moiety the activated carboxy group for effecting acylation]. Reaction is suitably performed in an inert solvent or diluent such as dichloromethane at e.g. ambient temperature. Alternatively hydroxy groups may be etherified, e.g. to introduce an epoxy bearing moiety of formula $-CH_2-\overset{O}{\widehat{CH-CH_2}}$ [the epoxy moiety providing the activated coupling group]

employing any of the various agents known in the art for such purpose, such as epichlorhydrin or epibromhydrin, e.g. in accordance with the general procedures described by Laumen et al. Loc. cit..

Cyclosporin starting materials for process step i) above may be prepared analogously to process step ii), e.g. for the production of a cyclosporin having a carboxy-substituted α-amino acid residue, e.g. at the 2- or 8-position:

iii) by reaction of a cyclosporin having an amino- or hydroxy-substituted α-amino acid residue, e.g. hydroxy-substituted α-amino acid residue at the 2-position or amino- or hydroxy-substituted α-amino acid residue at the 8-position, either a) with a dicarboxylic acid in which one of the carboxy groups present is in protected form, or b) with a dicarboxylic acid anhydride e.g. succinic anhydride, reaction in case a) being followed by deprotection of the carboxy group in the product cyclosporin.

Reaction step iii) may also be carried out employing essentially conventional procedures, e.g. in the presence of an acid binding agent such as 4-dimethylaminopyridine, in an inert organic solvent or diluent, at ambient or slightly elevated temperature. Where carboxy protecting groups are employed as in variant a) these may be entirely conventional and removed by entirely conventional technique.

Cyclosporin starting materials for process steps ii) and iii) having a hydroxy-substituted α-amino acid residue include the known cyclosporins: [Thr]^{2}-Cyclosporine and [(D)Ser]^{8}-Cyclosporine, the latter being disclosed and claimed e.g. in European

Patent No. 0 056 782, together with processes for its production in accordance with the general techniques of the total-synthetic method for the production of cyclosporins referred to above, or by fermentation technique. Other cyclosporins having a hydroxy-substituted α-amino acid residue, e.g. in the 8-position, may be prepared or obtained analogously and various further such Cyclosporins including [(D)Thr]^8-Cyclosporine, [Nva]^2-[(D)Ser]^8-Cyclosporine, and [Thr]^2-[(D)Ser]^8-Cyclosporine have been described and claimed in US Patent Application Ser. No. 713 259 (filed 19 March, 1985) = W. German Appn. No. P 3 509 809.0 (filed 19 March, 1985) = French Appn. No. 8 404 172 (filed 19 March, 1985) ≡ Australian Appn. No. 40 272/85 (filed 22 March, 1985) ≡ UK Appn. No. 8 507 270 (filed 20 March, 1985) ≡ New Zealand Appn. No. 211 526 (filed 21 March, 1985) ≡ South African Appn. No. 85/2195 (filed 22 March, 1985).

Preferred cyclosporins having an amino-substituted α-amino acid residue are those wherein the said amino acid residue is at the 8-position, cyclosporins wherein the residue at the 8-position is -(D)Lys- being especially preferred. Such cyclosporins may also be prepared in accordance with the general techniques of the total-synthetic method for the production of cyclosporins developed by R. Wenger, e.g.

iv) by deprotection of a cyclosporin having an amino-substituted α-amino acid residue at the 8-position said cyclosporin being in protected form, e.g. by deprotection of a cyclosporin wherein the residue at the 8-position is -(D)Lys- in N-ε-protected form; or

v) cyclising a straight chain undecapeptide having the sequence of the product cyclosporin, said undecapeptide being in free or protected form, e.g. undecapeptide comprising a -(D)Lys- residue in free or N-ε-protected form at the position corresponding to the 8-position of the product cyclosporin, and when required carrying out precess step iv).

Process steps iv) and v) may in particular be carried out in accordance with the general procedure hereinafter illustrated in example 1.

As will be appreciated from the description of process steps i), ii) and iii) above, the products of steps i) or ii) will generally comprise cyclosporins having an acylamino-, acyloxy- or alkoxy-substituted α-amino acid residue (i.e. α-amino acid residue having a side chain at the α-carbon atom comprising or bearing an acylamino-, acyloxy- or alkoxy- group), e.g. cyclosporin having an acyloxy- or alkoxy-substituted α-amino acid residue at the 2-position or acylamino-, acyloxy- or alkoxy-substituted α-amino acid residue at the 8-position, in which the activated coupling group is present on the acyl/alkyl moiety.

This may be more readily appreciated by reference to the following reaction schemes, illustrating the production of particular groups of cyclosporins in accordance with the general methods of process steps i) to v) above:

In the following formula Ia to Id, IIa to IId and III,

C represents the sequence -$\underset{3}{\text{Sar}}$-$\underset{4}{\text{MeLeu}}$-$\underset{5}{\text{Val}}$-$\underset{6}{\text{MeLeu}}$-$\underset{7}{\text{Ala}}$-, and

E represents the sequence -$\underset{9}{\text{MeLeu}}$-$\underset{10}{\text{MeLeu}}$-$\underset{11}{\text{MeVal}}$-.

Reaction step i)

a) $\boxed{A^1\text{-}B_a^1\text{-}C\text{-}D^1\text{-}E}$ (Ia)

in which

A^1 = -MeBmt-,

B_a^1 = -(O-acyl)-Thr- in which the acyl moiety bears a coupling group in non-activated form, for example a free carboxy group, e.g. -(O-hydroxysuccinyl)-Thr-, and

D^1 = -(D)Ala-:

↓ Activation, e.g. by reaction with a carboxy activating agent suitably in the presence of a coupling agent such as EDCI:

$\boxed{A^1\text{-}B_b^1\text{-}C\text{-}D^1\text{-}E}$ (IIa)

in which

A^1 and D^1 have the meanings given above, and

B_b^1 = -(O-acyl)-Thr- in which the acyl moiety bears an activated coupling group, for example an activated carboxy group, e.g. -(O-acyl)-Thr- in which the acyl moiety has the formula $ZO\text{-}CO\text{-}(CH_2)_2\text{-}CO\text{-}$ in which Z is a carboxy activating group.

b) $\boxed{A^2\text{-}B^2\text{-}C\text{-}D_a^2\text{-}E}$ (Ib)

in which

A^2 = -MeBmt- and B^2 = -αAbu- or -Nva-, or

A^2 = -dihydro-MeBmt-, and B^2 = -Val- and

D_a^2 = an acylamino substituted (D)α-amino acid residue, e.g. -(N-ε-acyl)-(D)Lys-, in which the acyl moiety bears a coupling group in non-activated form, for example a free carboxy group, e.g. -(N-ε-hydroxy-succinyl)-(D)Lys-:

$\downarrow$ Activation, e.g. by reaction with a carboxy activating agent, e.g. N-hydroxy succinimide, suitably in the presence of a coupling agent such as ECDI:

$$\text{A}^2\text{-B}^2\text{-C-D}_b^2\text{-E} \qquad \text{(IIb)}$$

in which

A^2 and B^2 have the meanings given above and

D_b^2 = an acylamino substituted (D)α-amino acid residue, e.g. -(N-ε-acyl)-(D)Lys-, in which the acyl moiety bears an activated coupling group, for example an activated carboxy group, e.g. -(N-ε-acyl)-(D)Lys- in which the acyl moiety has the formula $ZO-CO-(CH_2)_2-CO-$ in which Z has the meaning given above.

<u>Reaction step ii)</u>

a) $$\text{A}^1\text{-B}_a^3\text{-C-D}^1\text{-E} \qquad \text{(Ic)}$$

in which

A^1 and D^1 have the meanings given above, and

B_a^3 = -Thr-:

O-alkylation to introduce an alkyl moiety bearing an activated coupling group, e.g. epoxy group, e.g. by reaction with epichlorhydrin or epibromhydrin:

$$\boxed{A^1\text{-}B_b{}^3\text{-}C\text{-}D^1\text{-}E} \qquad \text{(IIc)}$$

in which

A^1 and D^1 have the meanings given above, and

$B_b{}^3$ = -(O-alkyl)-Thr- in which the alkyl moiety bears an activated coupling group, for example an epoxy group, e.g. -(O-epoxymethyl)-Thr-.

b) $$\boxed{A^2\text{-}B^2\text{-}C\text{-}D_a{}^3\text{-}E} \qquad \text{(Id)}$$

in which

A^2 and B^2 have the meanings given above and

$D_a{}^3$ = an amino substituted (D)α-amino acid residue, e.g. -(D)Lys-:

N-ε-acylation employing an acylating agent bearing an activating coupling group non-reactive with $-NH_2$, e.g. an acylating agent of formula $ZO\text{-}CO\text{-}(CH_2)_2\text{-}Y$ wherein Z has the meaning given above and Y is an activated coupling group non-reactive with $-NH_2$, e.g. a 2-pyridyl-dithio group:

$$\boxed{A^2\text{-}B^2\text{-}C\text{-}D_b^3\text{-}E} \qquad \text{(IId)}$$

in which A^2 and B^2 have the meanings given above, and

D_b^3 = an acylamino substituted (D)α-amino acid residue, e.g. -(N-ε-acyl)-(D)Lys-, in which the acyl moiety bears an activated coupling residue, e.g. -[N-ε-(3-(2-pyridyl)dithio-propion-1-yl)]-(D)Lys-.

Reaction step iii)

a) A cyclosporin of formula Ic as defined above:

↓ Acylation, e.g. by reaction with succinic anhydride:

A cyclosporin of formula Ia as defined above.

b) A cyclosporin of formula Id as defined above:

↓ Acylation, e.g. by reaction with succinic anhydride:

A cyclosporin of formula Ib as defined above.

Reaction step iv)

$$\boxed{A^2\text{-}B^2\text{-}C\text{-}D_a^4\text{-}E} \qquad \text{(Ie)}$$

in which

A^2 and B^2 have the meanings given above and

D_a^4 = an amino substituted (D)α-amino acid residue, e.g. -(D)Lys-, in protected form:

↓ Deprotection:

A cyclosporin of formula Id as defined above.

Reaction step v)

$H\text{-}D_a^5\text{-}E\text{-}A^2\text{-}B^2\text{-}C\text{-}OH$ (III)

in which
A^2 and B^2 have the meanings given above and
D_a^5 = an amino substituted (D) α-amino acid residue, e.g. -(D)Lys-, in free or -N-ε-protected form:

↓ Cyclisation in accordance with the method of R. Wenger:

A Cyclosporin of formula Id or Ie as defined above.

It may at this point be noted that the hydroxy group at the 3'-position in -MeBmt- and -dihydro-MeBmt- is of relatively low reactivity. Thus where processes described above involve reaction of cyclosporins having a hydroxy substituted α-amino acid residue at any one of positions 2 to 11, e.g. -Thr- in the 2-position, reaction with the hydroxy group of said residue will be in preference to reaction with the hydroxy group in -MeBmt- or -dihydro-MeBmt-, unwanted side reaction with the latter thus being readily avoidable.

In the formulae Ib, IIb, Id, IId, Ie and III above, A^2 and B^2 preferably represent -MeBmt- and -αAbu- respectively.

Whenever throughout the whole of the foregoing description, cyclosporins are referred to as having a specified residue at the 8-position, but the configuration of said residue is not recited, the (D)-configuration is preferred.

Cyclosporins having an activated coupling group described above as well as cyclosporins having an amino-substituted α-amino acid residue at the 8-position in which the amino substituent is in free or protected form, or is otherwise derivatised, e.g. acylated are novel and comprise a part of the present invention. The present invention accordingly provides:

1.1 A cyclosporin having an α-amino acid residue bearing an activated coupling group.

1.2 A cyclosporin according to 1.1 wherein the said α-amino acid residue is present at one of positions 2 through 11 inclusive.

1.3 A cyclosporin according to 1.2 wherein the said α-amino acid residue comprises an acylamino-, acyloxy- or alkoxy-substituted α-amino acid residue in which, the activated coupling group is present on the acylamino-, acyloxy- or alkoxy substituent.

1.4 A cyclosporin according to any one of 1.1 to 1.3 wherein the activated coupling group is an activated ester, activated dithio, or epoxy group.

1.5 A cyclosporin according to 1.3 wherein the said α-amino acid residue comprises: an acylamino substituted α-amino acid residue, wherein the acylamino substituent is substituted in the acyl moiety thereof by an activated carboxy or activated dithio group; an acyloxy substituted α-amino acid residue, wherein the acyloxy substituent is substituted in the acyl moiety thereof by an activated carboxy group; or an alkoxy substituted α-amino acid residue, wherein the alkoxy substituent is substituted by an epoxy group.

1.6 A cyclosporin according to any one of 1.3 to 1.5 wherein the said α-amino acid residue is an (O-acyl)-threonyl residue at the 2-position.

1.7 A cyclosporin according to 1.6 wherein the acyl moiety has the formula $ZO-CO-CH_2-CH_2-CO-$ wherein Z is a carboxy activating group.

1.8 A cyclosporin according to 1.2 wherein the said α-amino acid residue is present at the 5-, 6-, 7- or 8-position.

1.9 A cyclosporin according to 1.8 wherein the said α-amino acid residue is a (D)α-amino acid residue in the 8-position.

1.10 A cyclosporin according to 1.9 wherein the said α-amino acid residue is an acylamino substituted (D)α-amino acid residue in which the activated coupling group is present on the acylamino substituent.

1.11 A cyclosporin according to 1.10 wherein the activated coupling group is an activated carboxy or activated dithio group.

1.12 A cyclosporin having an amino substituted (D)α-amino acid residue at the 8-position the amino substituent being in free or protected form.

1.13 A cyclosporin having an acylamino substituted (D)α-amino acid residue at the 8-position wherein the acylamino substituent is substituted in the acyl moiety thereof by a free carboxy group.

1.14 A cyclosporin according to any one of 1.10 to 1.13 of formula

$$\begin{array}{c} \text{NH-X} \\ | \\ (CH_2)_4 \\ | \\ \text{-NH-CH-CO-} \\ \text{(D)} \end{array}$$

wherein X is hydrogen, an amino protecting group or an acyl group substituted by a free carboxy group or an activated coupling group, for example an activated carboxy or dithio group, e.g. an acyl group of formula

$$Y\text{-}CH_2\text{-}CH_2\text{-}CO\text{-}$$

wherein Y is carboxy, an activated carboxy group or an activated dithio group.

1.15 A cyclosporin of formula IIa, Ib, IIb, IIc, Id, IId or Ie as hereinbefore defined.

As hereinbefore discussed it has, in accordance with the present invention, now surprisingly been found that immunogenic conjugates comprising a carrier and a cyclosporin coupled by the agency of an activated coupling group, in particular, obtained employing cyclosporins having an activated coupling group as described above, e.g. as defined under 1.1 to 1.11, 1.14 or 1.15, enable, for the first time, the production of monoclonal antibodies capable of distinguishing between cyclosporins and metabolites thereof. Thus immunogenic conjugates comprising the reaction products of such cyclosporins as aforesaid as hapten component are capable of eliciting an antibody response in animals challenged, e.g. inoculated therewith, such that antibody producing cells, e.g. spleen or lymph-node cells, subsequently recoverable therefrom may be used for the preparation of hybridoma lines providing monoclonal antibodies capable of distinguishing between therapeutically administered cyclosporins, e.g. Cyclosporine, and metabolites thereof, in particular metabolites thereof in man, e.g. Cyclosporine <u>17</u> and Cyclosporine <u>18</u>. Such antigenic conjugates being hitherto unknown, the present invention further provides:

2.1 An immunogenic conjugate comprising a carrier coupled to a cyclosporin by the agency of an activated coupling group, for example comprising a carrier coupled to a cyclosporin having an α-amino acid residue bearing an activated coupling group, e.g. as hereinbefore described, in particular a cyclosporin as hereinbefore defined under any one of 1.1 to 1.11, 1.14 or 1.15 (formulae IIa, IIb, IIc or IId) above.

2.2 An immunogenic conjugate obtained or obtainable by coupling of a cyclosporin having an α-amino acid residue bearing an activated coupling group, e.g. as hereinbefore described, in particular a cyclosporin as hereinbefore defined under any one of 1.1 to 1.11, 1.14 or 1.15 (formulae IIa, IIb, IIc or IId) above.

2.3 An immunogenic conjugate, e.g. as defined under 2.1 or 2.2, capable of use in the production of a monoclonal antibody capable of distinguishing between a cyclosporin and a metabolite thereof, e.g. a monoclonal antibody as hereinafter described and, in particular, as hereinafter defined under any one of 3.1 to 3.10 below.

Suitable carriers for the immunogenic conjugates of the invention include any of those known and commonly employed in the art in particular high molecular weight polypeptides, especially proteins such as serum albumins, e.g. bovine serum albumin and chicken ovalbumin, immunoglobulins, in particular of the class IgG such as chicken or guinea pig IgG and synthetic polymers such as polyglutamic acid.

In addition the present invention provides a process for the production of an immunogenic conjugate as defined above, which process comprises:

vi) Coupling a carrier, e.g. as hereinabove described, bearing an activated coupling group with a cyclosporin having an α-amino acid residue bearing an appropriate co-reactive, e.g. hydroxy or amino, group, e.g. with [Thr]^2-Cyclosporine or [(D)Lys]^8-Cyclosporine, or coupling a carrier, e.g. as hereinabove described, with a cyclosporin having an α-amino

acid residue bearing an activated coupling group, e.g. as hereinbefore described, in particular a cyclosporin as hereinbefore defined under any one of 1.1 to 1.11, 1.14 or 1.15 (formulae IIa, IIb, IIc or IId) above.

The above process step is carried out by direct reaction of the cyclosporin component, i.e. without use of a coupling agent. Reaction is suitably effected by addition of the cyclosporin component dissolved in an appropriate inert diluent or carrier such as dimethyl formamide to a buffered preparation of the carrier, e.g. carrier protein, e.g. solution or supension in bicarbonate buffer, at ambient temperature. The obtained immunogenic conjugate is suitably purified by dialysis, e.g. against phosphate buffered saline.

The above described immunogenic conjugates, e.g. as defined under 2.1 to 2.3, may be employed to produce monoclonal antibodies by essentially standard techniques, e.g. via a stepwise procedure comprising: a) administration of an immunogenic conjugate, e.g. as defined under any one of 2.1 to 2.3 above, to an appropriate animal species; b) recovery of antibody producing, e.g. spleen or lymph-node, cells sensibilised to the immunogenic conjugate; c) immortalization of recovered cells, e.g. by fusion with an appropriate myeloma cell line to produce hybridoma cell lines; and d) selection of an immortalized cell, e.g. hybridoma line , producing monoclonal antibodies as required.

Step a) is suitably carried out using rats or mice, e.g. ♀ Balb/c mice as recipient, and administration of the immunogenic conjugate by s.c. or i.p. injection in an amount of from ca. 50 to 200, e.g. ca. 100 µg followed by booster injections, i.p., s.c.

or i.m., 14 to 21 days later. Mice showing high-titred antisera of appropriate isotype distribution, e.g. as determined by regular RIA and/or ELISA technique, are given further booster injections e.g. in accordance with the specific procedures hereinafter described in example 9, and antibody producing, e.g. spleen cells, collected [step b)]. Step c) may be performed in accordance with any of the techniques practiced in the art, e.g. using the method described by S. Fazekas et al., "J. Immunol. Methods" 35, 1-32 (1980), a preferred myeloma line being a mouse (Balb/C) line. In step d), growing myeloma lines are screened for antibody production against a cyclosporin, e.g. in a regular RIA system using a radiolabelled derivative thereof or in a regular ELISA system, e.g. as hereinafter described in example 9.

By application of the above procedures using the particular immunogenic conjugates of the present invention, it is possible to obtain monoclonal antibodies which exhibit a degree of specificity such that they are capable of distinguishing between individual cyclosporins differing from one another in only minor structural elements, e.g. presence of a single hydroxy group in place of a hydrogen atom. More importantly the present invention makes it possible, for the first time, to obtain monoclonal antibodies capable of distinguishing between cyclosporins, e.g. Cyclosporine, and metabolites thereof, in particular metabolites thereof in man. Thus monoclonal antibodies obtainable in accordance with the methods of the invention are found to be reactive with cyclosporins, e.g. Cyclosporine, while exhibiting relatively low cross-reactivity with metabolites thereof. Moreover employing immunogenic conjugates in accordance with the invention, e.g. as defined under 2.1 to 2.3 above, in which the cyclosporin hapten component corresponds to a selected "target" cyclosporin, the present invention enables the obtention of mono-

clonal antibodies capable of distinguishing between the "target" cyclosporin and structurally closely related metabolites, e.g. human metabolites, thereof. Thus starting from immunogenic conjugates obtained by coupling of a carrier with a cyclosporin having an activated coupling group at the 2-position, e.g. as defined under 1.6 above, and in which the α-amino acid residues at the remaining positions 1 and 3 to 11 are the same as those in Cyclosporine, it is possible to prepare monoclonal antibodies reactive with Cyclosporine as "target" cyclosporin in preference to metabolites thereof in man, e.g. Cyclosporines 1, 8, 9, 10, 16, 17, 18 and/or 21, in particular 17 and/or 18 and especially 17. Similarly, starting from immunogenic conjugates obtained by coupling of a carrier with a cyclosporin having an activated coupling group at the 5-, 6-, 7- or 8-position, e.g. as defined under 1.8 above, in particular the 8-position, e.g. as defined under any one of 1.9, 1.11, 1.14 or 1.15 (formulae IIb or IId) above, and in which the residues at the remaining positions, e.g. 1 to 7 and 9 to 11, correspond to those in Cyclosporine, dihydro-[Val]^2-Cyclosporine or [Nva]^2-Cyclosporine, monoclonal antibodies may be prepared reactive with Cyclosporine, dihydro-[Val]^2-Cyclosporine or [Nva]^2-Cyclosporine as "target" cyclosporin, in preference to metabolites thereof, e.g. metabolites thereof in man, such as, in the case of Cyclosporine, those recited immediately above.

Monoclonal antibodies obtainable in accordance with the methods of the invention are, in particular, capable of distinguishing between "target" cyclosporins and metabolites thereof exhibiting structural transformation of the α-amino acid residue at the 1-position, e.g. metabolites which differ from the non-metabolised cyclosporin from which they are derived by substitutional or other chemical modification of the -MeBmt- or

-dihydro-MeBmt- residue at the 1-position, in particular exhibiting structural transformation at a terminal position in the residue at the 1-position, e.g. comprising hydroxylation at of the terminal (C^9) -MeBmt- methyl group, as in the case of monoclonal antibodies described in the accompanying example 9, which are reactive with Cyclosporine while having relatively low cross-reactivity with its metabolite Cyclosporine 17. In so far as such metabolic transformation of cyclosporin is of especial significance, e.g. as characteristic of major metabolites in man, as in the case of Cyclosporins 17 and 18, ability of monoclonal antibodies obtainable in accordance with the methods of the invention to distinguish between cyclosporins and metabolites thereof exhibiting such transformation is in particular to be noted.

Cross-reactivity with metabolites, e.g. as described above, is preferably ca. 5 % or less, more preferably ca. 3 % or less, more preferably ca. 2 % or less, of reactivity with the non-metabolised cyclosporin, e.g. as measure by RIA or ELISA, e.g. competitive ELISA, technique, suitably employing a buffer e.g. of ca. pH 6 to 8, in particular 7 to 8, and appropriately also containing a minor amount, e.g. 0.01 to 0.1 % e.g. 0.01 to 0.05 %, of a non-ionic surfactant or tenside such as Tween, for example phosphate buffered saline at pH 7.5 and containing 0.03 % surfactant, e.g. Tween 20. Thus monoclonal antibodies obtainable in accordance with the methods of the invention and reactive with Cyclosporine exhibit a distinction in IC_{50} ratio for Cyclosporine 17 as compared with Cyclosporine, measured by competitive ELISA technique under conditions as set forth above of the order of 35 fold or greater.

Monoclonal antibodies obtainable in accordance with the methods of the invention are also characterised by high affinity for the

"target" cyclosporin, e.g. Cyclosporine. Thus preferred monoclonal antibodies in accordance with the invention will exhibit an affinity constant [equilibrium dissociation constant] in respect of the "target" cyclosporin, e.g. Cyclosporine, of the order of 10^{-9} mol/L or less, preferably 10^{-10} mol/L or less,e.g. at normal RIA temperatures (ca. 4 to 37 °C) as determined by standard methods, e.g. in accordance with the method described by Müller et al., Methods in Enzymology, 92, 589 - 601 (1983).

The present invention further permits the ready obtention of monoclonal antibodies of the class IgG, e.g. of the sub-class IgG_1. In so far as such antibodies are especially suited to use in diagnostic/assay kits, e.g. as described below, these are preferred.

Monoclonal antibodies as described above, as well as hybridoma lines producing them are entirely novel and, as will be appreciated from the foregoing description of their general and specific properties, well adapted for use in diagnostic/assay kit systems, e.g. for monitoring of cyclosporin drug plasma-blood levels in patients receiving cyclosporin therapy. The present invention accordingly also provides:

3.1 A monoclonal antibody capable of distinguishing between a cyclosporin, e.g. a predetermined cyclosporin, and a metabolite thereof, in particular at least one metabolite thereof in man, especially at least one major metabolite thereof in man.

3.2 A monoclonal antibody according to 3.1 reactive with a cyclosporin, e.g. a predetermined cyclosporin, and exhibiting relatively low cross-reactivity with a metabolite thereof, in particular at least one metabolite

thereof in man, especially at least one major metabolite thereof in man.

3.3 A monoclonal antibody according to 3.1 or 3.2 wherein the cyclosporin is Cyclosporine, dihydro-[Val]^2-Cyclosporine or [Nva]^2-Cyclosporine, especially Cyclosporine.

3.4 A monoclonal antibody according to any one of 3.1 to 3.3 wherein the metabolite is a metabolite exhibiting structural transformation of the α-amino acid residue at the 1-position, in particular at a terminal position on the residue at the 1-position, e.g. exhibiting terminal hydroxylation of the α-amino acid residue -MeBmt- at the 1-position.

3.5 A monoclonal antibody according to 3.4 wherein the cyclosporin is Cyclosporine and the metabolite is Cyclosporin <u>1</u>, <u>8</u>, <u>9</u>, <u>10</u>, <u>16</u>, <u>17</u>, <u>18</u> or <u>21</u>, especially Cyclosporine <u>17</u> or <u>18</u>, most especially Cyclosporine <u>17</u>.

3.6 A monoclonal antibody according to any one of 3.2 to 3.5 wherein cross reactivity with the metabolite is of the order of ca. 5 % or less, preferably 3 % or less, more preferably 2 % or less, e.g. as measured by RIA or ELISA technique, for example under conditions as hereinbefore set forth.

3.7 A monoclonal antibody according to any one of 3.1 to 3.6 wherein the affinity constant with respect to the (predetermined) cyclosporin, e.g. Cyclosporine, is of the order of 10^{-9} mol/litre or less, preferably 10^{-10} mol/litre or less, e.g. as measured under conditions as hereinbefore set forth.

3.8 A monoclonal antibody according to any one of 3.1 to 3.7 of the class IgG.

3.9 A monoclonal antibody, e.g. according to any one of 3.1 to 3.8, obtained or obtainable by:

a) coupling of a cyclosporin having an α-amino acid residue bearing an activated coupling group, e.g. as hereinbefore described, in particular as hereinbefore defined under any one of 1.1 to 1.11, 1.14 or 1.15 (formulae IIa, IIb, IIc or IId) above, to a carrier to obtain an immunogenic conjugate;

b) administration of said immunogenic conjugate to an appropriate animal species to effect immunogenic challenge, and recovery of antibody producing cells sensitised to said conjugate;

c) immortalisation of said antibody producing cells, e.g. by fusion with an appropriate myeloma cell line; and

d) recovery of monoclonal antibody from a selected immortalised cell line, e.g. hybridoma cell line, thus established.

3.10 A monoclonal antibody, e.g. according to any one of 3.1 to 3.8 obtained or obtainable by:

a) recovery of antibody producing cells sensitised to an immunogenic conjugate according to any one of 2.1 to 2.3 above;

b) immortalisation of said antibody producing cells, e.g. by fusion with an appropriate myeloma cell line, and

c) recovery of the required monoclonal antibody from a selected immortalised cell line, e.g. hybridoma cell line, thus established.

4.1 A hybridoma cell line producing a monoclonal antibody according to any one of 3.1 to 3.8 above.

4.2 A hybridoma cell line obtained or obtainable in accordance with steps a) to c) of 3.9 above or steps a) and b) of 3.10 above.

As will be appreciated, monoclonal antibodies in accordance with the invention may distinguish between any given cyclosporin, e.g. Cyclosporine, and a plurality of its metabolites, e.g. exhibit low-cross reactivity with respect to more than one of its metabolites.

In addition to the foregoing the present invention also provides:

vii) A method for the production of a monoclonal antibody as defined under any one of 3.1 to 3.8 above, which method comprises culturing a hybridoma cell line producing such antibody and recovering the antibody thus produced; and

viii) A method for the production of a hybridoma cell line producing a monoclonal antibody as defined under any one of 3.1 to 3.8 above, which method comprises immortalizing an antibody producing cell, e.g. spleen or lymph-node cell, producing such antibody, e.g. by fusion with an appropriate myeloma cell line.

The above process steps may be performed in accordance with now standard techniques, e.g. as hereinabove described, or as described in the accompanying examples, preferred myeloma cell lines for use in process viii) being a mouse (Balb/C) myeloma cell line.

In accordance with a yet further aspect of the present invention it has also surprisingly been found that cyclosporins having a -(D)Lys- residue at the 8-position, i.e. as defined under 1.14 or 1.15 (formula Id) above, as well as derivatives in which the N-ε-atom thereof is further derivatised, exhibit e.g. cell binding characteristics which parallel those of the corresponding "parent" cyclosporin (e.g. the corresponding cyclosporin having -(D)Ala- at the 8-position) to a surprising and remarkable degree. This finding is of especial significance since the N-ε-nitrogen atom of -(D)Lys- provides an ideal position at which labelling may be effected, e.g. at which label or tracer groups may be introduced. Such labelled cyclosporins provide a further key tool for study of the mechanism of action of "parent" cyclosporins (e.g. in the case of [(D)Lys]^{8}-Cyclosporine of Cyclosporine) and/or for identifying binding sites of the "parent" cyclosporin, e.g. in _in vitro_ tissue culture preparations. Thus radioactively labelled derivatives, e.g. ^{125}I labelled derivatives, are useful for rapid autoradiography of tissues, e.g. as in kidney micro-autoradiography.

In addition labelled, e.g. radioactively or fluorescently labelled, derivatives obtainable from cyclosporins having a -(D)Lys- residue at the 8-position provide ideal components for use e.g. in RIA and FIA diagnostic kits. [(D)Lys]^{8}-cyclosporins thus provide a means for the ready obtention of labelled analogues of the "parent" cyclosporin having equivalent binding

properties, e.g. in relation to monoclonal antibodies to the parent cyclosporin, e.g. as obtained in accordance with the present invention or as hereinabove defined, and hence emminently useful as diagnostic/assay kit component or co-component.

Accordingly the present invention also provides:

5.1 A labelled derivative of a cyclosporin wherein the residue at the 8-position is -(D)Lys-; in particular

5.2 A labelled derivative of a cyclosporin of formula Id as defined above.

By the term "labelled derivative" as used herein is meant a derivative bearing a tracer or marker atom or group, e.g. enabling or facilitating quantitative assay or location of said derivative. Such derivatives include derivatives, e.g. wherein one or more atoms of the -(D)Lys- residue functions as a tracer or marker atom, e.g. radioactive atom, as well as derivatives wherein a tracer or marker group is attached to the N-ε-atom of the -(D)Lys- residue either by direct linkage of the tracer or marker group to said N-ε-atom or by linkage of the tracer or marker group to said N-ε-atom via an intervening linking moiety. Examples of labelled derivatives include radioactively labelled derivatives, fluorescent and chemiluminescent derivatives and derivatives suitable for photoaffinity labelling, i.e. provided with a substituent which will react with a protein to which the cyclosporin is bound on illumination. Radioactively labelled derivatives as aforesaid include derivatives wherein the N-ε-atom of the -(D)Lys- residue at the 8-position attaches to e.g. an ^{125}I labelled p-OH-phenyl-propionyl residue. Fluorescent and chemiluminescent derivatives as aforesaid include derivatives

wherein the N-ε-atom of the -(D)Lys- residue at the 8-position attaches to a fluorescent group, such as a dansyl or rhodamine group, or chemiluminescent group such as an acridinium ester group, e.g. as described in Clin. Chem. 29, 8, pp 1474 - 1479 (1983). A particular group of cyclosporins as defined under 5.1 and 5.2 above are accordingly

5.3 those wherein the residue at the 8-position is a residue of formula

$$\begin{array}{c} \text{NH-Q} \\ | \\ (CH_2)_4 \\ | \\ \text{-NH-CH-CO-} \\ \text{(D)} \end{array}$$

wherein Q is or comprises a tracer or marker group, in particular radioactively labelled, fluorescent or chemiluminescent group, e.g. as specifically described above.

Labelled derivatives as aforesaid may be prepared analogously to process steps iv) and v) above, e.g. employing starting materials in which the -(D)Lys- residue at the 8-position is pre-labelled. Alternatively they may be prepared by introduction of an appropriate labelling substituent, e.g. at the N-ε-atom of the -(D)Lys- residue at the 8-position. Thus fluorescently labelled derivatives may be prepared by coupling of a fluorescent moiety to the N-ε-atom e.g. by N-ε-dansylation. Similarly radioactively labelled derivatives may be prepared by coupling of a radioactively labelled substituent, e.g. ^{125}I labelled p-OH-phenylpropionyl, to the N-ε-atom. In the latter case the substituent

may either be in labelled form prior to introduction or may be labelled subsequent to introduction. For example the N-ε-atom of the lysine residue in the 8-position may either be reacted directly with ^{125}I-labelled p-OH-phenyl-propionic acid or with unlabelled p-OH-phenyl-propionic acid and the obtained N-ε-amide subsequently labelled in the p-OH-phenyl moiety with ^{125}I. Coupling may be effected in accordance with standard techniques known in the art for example by reaction with p-OH-phenyl-propionic acid (labelled or unlabelled) in the form of its N-hydroxy-succinimide ester.

^{125}I labelled-p-OH-phenyl-propionic acid may itself be prepared by the chloramine T-method [Hunter and Greenwood, Nature, 194, 495 (1962)]. Where labelling is effected subsequent to coupling this may be carried out using the chloramin T-method or the iodogen-method [Good, J.Clin.-Chem.Clin.Biochem., 19, 1051 (1981)]. Derivatives of the cyclosporins of the invention which are susceptible to labelling, e.g. as described above, for example derivatives wherein the N-ε-atom of -(D)Lys- at the 8-position is substituted by a group, such as p-OH-phenyl-propionyl, susceptible to ^{125}iodination, are immediate precursors of the labelled derivatives of the invention and are also to be understood as being within the purview of the present invention.

In accordance with the foregoing the present invention also provides:

ix) A process for the production of a labelled derivative as defined under any one of 5.1 to 5.3 above, in free or protected form, which process comprises labelling the corresponding unlabelled free or protected cyclosporin, e.g. introducing a labelling substituent, for example as

hereinbefore described, at the N-ε-atom of the -(D)Lys-residue at the 8-position thereof, and when required carrying out process step iv) above.

It may at this point additionally be noted, that the cyclosporins having a free amino group as defined under 1.12 above, for example the compound [(D)Lys]^8-Cyclosporine, also possess pharmaceutical, in particular immunosuppressive, anti-inflammatory and anti-parasitic (e.g. anti-malarial and anti-coccidiomycotic), activity, as can be demonstrated in standard *in vivo* and *in vitro* tests, for example in the various test methods described in European Patent No. 0 056 782.

In accordance with a yet further aspect of the present invention it has been found that immunogenic conjugates in which the carrier molecule is coupled to a cyclosporin as hapten via the residue at the 5-, 6-, 7- or 8-position, in particular the 8-position, including such conjugates as defined under 2.1 to 2.3 above, as well as conjugates obtained by coupling of a cyclosporin bearing an amino acid having a reactive group at any one of these positions other than an activated coupling group to a carrier by means of a coupling agent, are capable of generating regular polyclonal antisera of higher specificity than hitherto obtainable e.g. employing conjugates obtained by coupling of a carrier to -(Thr)^2- in (Thr)^2-Cyclosporine.

(By the term "reactive group" as used above is to be understood any group which permits or enables coupling with a carrier, e.g. polypeptide or other appropriate macromolecule. Generically the term thus embraces activated coupling groups as hereinbefore defined as well as other groups capable of reaction, e.g. free amino, carboxy or hydroxy groups)..

Immunogenic conjugates comprising a cyclosporin as hapten, linked to a carrier via the α-amino acid residue at the 5-, 6-, 7- or 8-position are novel as such. Preferred cyclosporins providing the hapten moiety of such conjugates are in particular those having an activated coupling group as defined under 1.8 to 1.11, 1.14 and 1.15 (formulae IIb and IId) above.

The present invention accordingly also provides:

2.4 An immunogenic conjugate comprising a carrier coupled to a cyclosporin via the α-amino acid residue at position 5-, 6-, 7- or 8- of said cylosporin.

2.5 An immunogenic conjugate comprising a carrier coupled with a cyclosporin having an activated coupling group as defined under any one of 1.8 to 1.11, 1.14 or 1.15 (formulae IIb and IId) above.

2.6 An immunogenic conjugate comprising a carrier coupled with a cyclosporin having a free amino or carboxy group as defined under any one of 1.12 to 1.14 and 1.15 (formulae Ib and Id) above, in particular a cyclosporin as defined under 1.14 above or of formula Id as defined above.

3.11 A monoclonal antibody according to 3.9 above, characterised in that the cyclosporin employed at step a) is a cyclosporin as defined under 2.5 above.

6.1 Antibody reactive with a cyclosporin (including polyclonal antiserum containing antibodies reactive with a cyclosporin) generated in response to an immunogenic conjugate as defined under any one of 2.4 to 2.6 above.

6.2 Antibody according to 6.1 reactive with Cyclosporine, dihydro-[Val]^2-Cyclosporine or [Nva]^2-Cyclosporine, especially Cyclosporine.

Immunogenic conjugates as defined under 2.5 may be prepared in accordance with the methods of process step vi) above. Immunogenic conjugates as defined e.g. under 2.6 may be prepared by methods as hereinbefore described or by a process comprising:

x) Coupling a carrier to a cyclosporin having an α-amino acid residue bearing a reactive group (i.e. α-amino acid residue having a side chain at the α-carbon atom comprising or bearing a reactive group), e.g. a cyclosporin bearing a free amino or carboxy group as defined under any one of 1.12 to 1.14 and 1.15 (formulae Ib and Id) above.

The above process step may be effected in accordance with techniques known in the art, by linkage of the carrier to the cyclosporin via the intermediary of a coupling reagent. Thus in the case of cyclosporins having a -(D)Lys- residue at the 8-position, conjugates may be obtained by linkage of the carrier, e.g. polypeptide, for example immunoglobulin, to the -(D)Lys-N-$\mathcal{E}$-atom, employing the carbodiimide procedure [Kellie et al., "Steroid Immunology", ed. Cameron et al., Alpha. Omega, Cardiff, 1975] or by Mannich reaction employing formaldehyde as the coupling reagent.

Suitable carriers include those of the type already referred to in relation to the preparation of conjugates for the production of monoclonal antibodies, in particular high molecular weight polypeptides, especially proteins such a serum albumins, immunglobulins and synthetic polymers such as polyglutamic acid.

Regular polyclonal antisera as defined under 6.1, while lacking the fine specificity of monoclonal antibodies as hereinbefore described and defined, are also useful, e.g. as diagnostic/assay kit components, in particular having regard to their improved specificity with respect to cyclosporins as compared with such antisera known from the art. They may be prepared employing essentially conventional techniques, e.g. by a process comprising:

xi) Elliciting an immune response in an appropriate animal species by administration of an immunogenic conjugate as defined under any one of 2.4 to 2.6 above and recovering antisera thus generated.

The above defined process step xi) may be performed e.g. by administration of the immunogenic conjugate to e.g. a mouse, sheep or chicken, e.g. by injection. After an appropriate incubation period the antiserum is recovered and is suitably lyophylised, e.g. for later use in kits, for example as hereinafter described. The period of incubation is suitably chosen to give an antiserum titre of greater than 1 : 2,000, e.g. in the range of from about 1 : 7,000 to about 1 : 10,000, in e.g. regular RIA.

As previously indicated the monoclonal antibodies and polyclonal antisera, as well as labelled cyclosporins of the invention are all of particular utility as components of diagnostic/assay kit systems, e.g. immuno assay kits.

Accordingly, in a yet further aspect the present invention provides:

7. An immuno assay kit or system, e.g. RIA or FIA kit or system, for cyclosporine assay, for example for the assay of a cyclosporin, e.g. Cyclosporine, in subjects receiving cyclosporin, e.g. Cyclosporine, therapy, said kit or system comprising:

   A) Antibody or antiserum as defined under any one of 3.1 to 3.11 or 6.1 to 6.2 above, in particular a monoclonal antibody as defined under any one of 3.1 to 3.11 above, and/or

   B) a labelled derivative of a cyclosporin as defined under any one of 5.1 to 5.3 above,

   as component of said kit or system.

Kits as defined under 7 are useful for diagnostic purposes, e.g. for determining quantities of a cyclosporin present in blood, blood plasma or urine, e.g. as a means of establishing an appropriate dosaging regimen for patients receiving cyclosporin therapy. Such kits provide an assay means for cyclosporins, e.g. Cyclosporine, of hitherto unmatched sensitivity.

Kits, e.g. RIA and FIA, kits in accordance with the invention may be of entirely conventional type for use in accordance with conventional RIA and FIA assay techniques. Thus RIA kits will suitably comprise in addition to antiserum or antibody, e.g. A) above, an appropriate labelled cyclosporin derivative, e.g. B) above, and C) cyclosporin standard. The labelled cyclosporin derivative will be complementary to the cyclosporin to be assayed. Suitably it will be a labelled derivative as defined under B) above, e.g. where Cyclosporine is to be assayed it will

suitably be a labelled derivative of [(D)Lys]^{8}-Cyclosporine. However it may also be any other labelled complementary cyclosporin, for example where Cyclosporine is to be assayed, tritiated Cyclosporine. The cyclosporin standard C) will generally be a solution or the like comprising a known quantity of the cyclosporin to be assayed.

In use, e.g. lyophilised, antiserum/antibody is dissolved and incubated together with e.g. component B) and with either the sample to be assayed or component C). Incubation is preferably effected with cooling e.g. at 4°C. The pH of the incubating mixture is preferably kept in the range of from about 5 to 8, e.g. at about pH 7 or 8, preferably with the aid of a buffering agent such as a citrate or tris buffer.

Incubation conveniently lasts for at least 2 hours, e.g. from about 6 to about 12 hours. After incubation the fraction of e.g. component B) bound to the antibody is separated from the unbound fraction, e.g. by the use of charcoal such as dextran-coated charcoal. The unbound fraction adsorbs onto the charcoal and may then be separated by filtration or by centrifugation. The amount of radioactivity in one fraction is then measured by standard techniques, e.g. by liquid scintillation counting after the addition of a secondary solute. The proportion of component B) bound to the antibody is inverseley proportional to the amount of cyclosporin in the unknown plasma sample. For quantitive analysis, it is usual to prepare a standard calibration curve by analysing solutions of the cyclosporin of known concentration.

FIA kits in accordance with the invention may be e.g. of the kind wherein antibodies are bound to a light scavenger and which depend upon competition between a fluorescent cyclosporin (e.g. a

fluorescently labelled derivative in accordance with the invention) and the antibody.

Alternatively assay kits/systems as defined under 7 above may be based on any of the conventional ELISA systems known in the art.

The following examples are illustrative of the present invention:

EXAMPLE 1: Preparation of [(D)Lys^{8}]-Cyclosporine

a) A solution of 6.4 g H-MeLeu-MeLeu-MeVal-OBzl maleinate in CH_2Cl_2 (200 ml) and H_2O (100 ml) is adjusted to pH 8 using solid K_2CO_3. After extracting 2x, each time with CH_2Cl_2 (200 ml), the organic phase is dried over Na_2SO_4 filtered and evaporated to dryness to yield free H-MeLeu-MeLeu-MeVal-OBzl as a crystalline residue.

b) (N-$\mathcal{E}$-BOC)-FMOC-(D)Lys (6.25 g) is dissolved in $CHCl_3$ (100 ml) and N-methylmorpholine (2.95 g) is added with stirring. After cooling the solution to -20°, pivaloyl chloride (1.75 g) is added dropwise, and the reaction mixture is stirred for 6 hours at -20°. A solution of H-MeLeu-MeLeu-MeVal-OBzl (6.34 g) in $CHCl_3$ (20 ml) is added to the anhydride solution dropwise and stirred for 17 hours at -20° to complete reaction. After diluting the $CHCl_3$ solution with further $CHCl_3$ (200 ml), the mixture is shaken with saturated $NaHCO_3$ solution (100 ml). The organic phase is dried over Na_2SO_4, filtered and the solvent evaporated to dryness.

The obtained oily residue is purified chromatographically, using 25x the amount of silica gel (particle size 0.063 - 0.20 mm) and methylene chloride with additional 3 % methanol as eluant: $[\alpha]_D^{20}$ = -114.2° (c = 1.0 in $CHCl_3$).

c) (N-$\mathcal{E}$-BOC)-FMOC-(D)Lys-MeLeu-MeLeu-MeVal-OBzl (8.8 g) dissolved in absolute ethanol (400 ml) is hydrogenated with 10 % palladium/C catalyst (0.6 g) in a Gastar hydrogenator until the theoretical quantity of H_2 is taken up (214 ml). After evaporating off the solvent, the residue is purified chromatographically using 50x the quantity of silica gel

(0.063 - 0.20 mm) and methylene chloride plus 7 % methanol as eluant: $[\alpha]_D^{20}$ = - 129.1 ° (c = 1.0 in $CHCl_3$).

d) (N-ε-BOC)-FMOC-(D)Lys-MeLeu-MeLeu-MeVal-OH (7.1 g) and H-MeBmt-αAbu-Sar-MeLeu-Val-MeLeu-Ala-OBzl (7.4 g) are dissolved in methylene chloride (100 ml), and N-methylmorpholine (1.72 g) and Castro reagent ($Bt\text{-}OP(NMe_2)_3^+PF_6^-$) (5.6 g) are added at room temperature (25°). The reaction mixture is stirred for 3 days at room temperature, then the solution is diluted with methylene chloride (200 ml) and shaken with saturated $NaHCO_3$ solution (100 ml). The organic phase is dried over Na_2SO_4, filtered and evaporated to dryness. The obtained oily residue is purified chromatographically on silica gel (500 g) (0.06-0.20) using methylene chloride plus 3 % methanol as eluant: $[\alpha]_D^{20}$ = -143.8° (c = 1.0 in $CHCl_3$).

e) (N-ε-BOC)-FMOC-(D)Lys-MeLeu-MeLeu-MeVal-MeBmt-αAbu-Sar-MeLeu-Val-MeLeu-Ala-OBzl (5.54 g) are stirred for a total of 4 hours at room temperature in a solution of methylene chloride (50 ml) and piperidine (10 ml). The solvent is evaporated and the obtained oil chromatographed on Sephadex LH20 (300 g) using methylene chloride plus 3 % methanol as eluant: $[\alpha]_D^{20}$ = -165.2° (c = 1.0 in $CHCl_3$).

f) 0.2N NaOH (24 ml) is added to (N-ε-BOC)-H-(D)Lys-MeLeu-MeLeu-MeVal-MeBmt-αAbu-Sar-MeLeu-Val-MeLeu-Ala-OBzl (6.48 g) dissolved in ethanol (75 ml). After 7 hours the solution is adjusted to pH 4 by dropwise addition of 2N HCl with cooling. After evaporation of the solvent, the obtained residue is shaken in CH_2Cl_2 (200 ml) and saturated $NaHCO_3$ (200 ml) solution. After extraction of the aqueous phase 2x,

each time using methylene chloride (200 ml), the organic phase is dried over Na_2SO_4, filtered off and evaporated. The product is purified chromatographically on silica gel (300 g) (0.06-0.20 mm) using methylene chloride plus 20 % methanol as eluant: $[\alpha]_D^{20}$ = -169.9° (c = 1.0 in $CHCl_3$).

g) [Process step v)]

Dimethylaminopyridine (147 mg) is added with stirring to (N-ε-BOC)-H-(D)Lys-MeLeu-MeLeu-MeVal-MeBmt-αAbu-Sar-MeLeu-Val-MeLeu-Ala-OH (413 mg) dissolved in methylene chloride (2000 ml). Propane phosphonic acid anhydride [(0.19 g) 50 % solution in CH_2Cl_2] is added and the reaction mixture is stirred for 24 hours at 25°. The obtained solution is washed with saturated $NaHCO_3$ solution (200 ml), the organic phase is dried over $NaSO_4$, filtered off and evaporated, and purified chromatographically on silica gel (300 g) (0.062-0.20) using methylene chloride plus 5 % methanol as eluant: $[\alpha]_D^{20}$ = -198.3 (c = 1.0 in $CHCl_3$).

h) [Process step iv)]

[(N-ε-BOC)-(D)Lys]^8-Cyclosporine (842 mg) is cooled to -20° with trifluoroacetic acid (25 ml) and stirred together for 4 hours at -20°. The reaction solution is mixed with ice, sat. K_2CO_3 (10 ml) and extracted 3x with methylene chloride (200 ml). The organic phase is dried over Na_2SO_4, filtered and the solvent is evaporated. The obtained crude product is chromatographed on Sephadex LH20 (200 g) using methylene chloride plus 1 % methanol as eluant to yield the title compound [(D)Lys]^8-Cyclosporine: $[\alpha]_D^{20}$ = -204.3° (c = 1.0 in $CHCl_3$).

The product compound may also be converted into salt form in accordance with standard techniques. Typical salts include [(D)Lys]^8-Cyclosporine hydrochloride: $[\alpha]_D^{20}$ = - 203 ° (c = 1.0 in $CHCl_3$), and [(D(Lys]^8-Cyclosporine trifluoroacetate: $[\alpha]_D^{20}$ = - 203 ° (c = 1.0 in $CHCl_3$).

EXAMPLE 2: Preparation of [(N-ε-hydroxysuccinyl)-(D)Lys]^8-Cyclosporine: [Process step iii)]

255 mg of [(D)Lys]^8-Cyclosporine produced in accordance with example 1 are dissolved in 20 ml pyridine and 36 mg succinic anhydride are added. The obtained solution is stirred for ca. 14 hrs. at room temperature and the pyridine fully evaporated under vacuum at max. 40°C. The obtained oily residue is chromatographed on 55 g Sephadex LH 20 employing methylene chloride + 2 % methanol and collected in 10 ml fractions. The pure title compound is obtained from fractions 15 through 23.

NMR spectroscopy shows succinyl protons at 2.50 and 2.70 ppm (broad signals) and a signal for $-CH_2-NH-COCH_2CH_2COOH$ at 3.25 ppm.

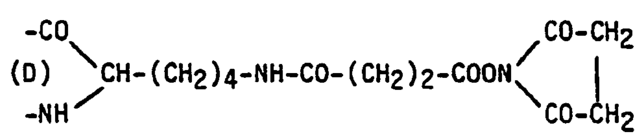
EXAMPLE 3: Preparation of [(O-hydroxysuccinyl)-Thr]^2-Cyclosporine: [Process step iii)]

6.05 g [Thr]^2-Cyclosporine is dissolved in 20 ml pyridine and 3.66 g 4-dimethylamino-pyridine and 1.5 g succinic anhydride are added at 75 ° C. The reaction mixture is stirred for 4 hours at 75 ° C and then diluted with 500 ml CH_2Cl_2, washed 5 x, each time with 50 ml 2N HCl, and 1x with 150 ml H_2O. The organic phase is extracted, dried over Na_2SO_4 and evaporated and purified chromatographically using 250 g silica gel (0.040 - 0.062 mm) with acetic acid as eluant.

EXAMPLE 4: Preparation of [(N-ε-succinimidooxysuccinyl)-(D)Lys]^8-Cyclosporine: [Process step i)]

50 mg of [(N-ε-hydroxysuccinyl)-(D)Lys]^8-Cyclosporine produced in accordance with example 2, 14 mg N-ethyl-N'-(dimethylamino-propyl)-carbodiimide HCl, 23.8 mg N-hydroxysuccinimide and 24.6 mg triethylamine are stirred for 2 hrs. at room temperature in 2 ml methylene chloride to yield a clear colourless solution. The obtained solution is diluted with 50 ml methylene chloride and 10 ml H_2O and 1N HCl is added dropwise until pH 6. A two phase mixture develops and this is shaken thoroughly between each addition of HCl. The organic phase is finally shaken with 10 ml dilute $NaHCO_3$ solution and dried over Na_2SO_4, filtered and the solvent evaporated off to yield the title compound.

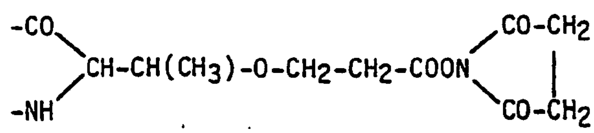
The NMR spectrum exhibits succinyl protons at 2.50 and 2.75 ppm (J = 5 cps) and N-succinimido protons at 2.18 and 2.19 ppm (2S). The residue at the 8-position has the following structure:



EXAMPLE 5: Preparation of [(O-succinimidooxysuccinyl)-Thr]^2-Cyclosporine: [Process step i)]

54 mg triethylamine, 98 mg N-hydroxysuccinimide and 102 mg N-ethyl-N'-(3-dimethylaminopropyl)-carbodiimide are added to a solution of 200 mg of [(O-hydroxysuccinyl)-Thr]^2-Cyclosporine produced in accordance with example 3 in 10 ml methylene chloride, addition being effected at 20 °C with rigorous exclusion of moisture. The reaction mixture is stirred for

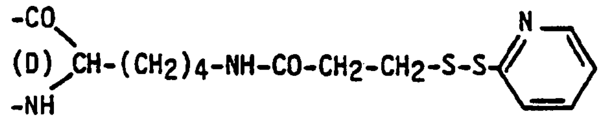
6 hours at room temperature, diluted with 200 ml methylene chloride and shaken with 50 ml H_2O. The aqueous phase in adjusted to pH 5 - 6 by drop-wise addition of 1N HCl and shaken. The aqueous phase is extracted with 100 ml methylene chloride and the organic phases washed with 0.1N $NaHCO_3$, dried over Na_2SO_4, filtered and evaporated. The residue is purified chromatographically using 110 g silica gel with ethylacetate as eluant to yield the title compound: $[\alpha]_D^{20} = -178°$ (c = 1.0 in $CHCl_3$). ^1H-NMR in $CDCl_3$ shows succinimido protons at 2.80 as a singlet and succinyl protons at 2.60 ppm as a multiplet. The residue at the 2-position has the following structure:



EXAMPLE 6: <u>Preparation of [(N-(3-(2-pyridyl)dithio)propion-1-yl)-(D)Lys]^8-Cyclosporine: [Process step ii)]</u>

35 mg succinyl-3-[(2-pyridyl)dithio]propionate are added to a solution of 126 mg [(D)Lys]^8-Cyclosporine in 10 ml methylene chloride at 20 °C and with rigorous exclusion of moisture. The reaction mixture is stirred for 6 hours at room temperature, diluted with 200 ml methylene chloride and shaken with 50 ml saturated $NaHCO_3$. The aqueous phase is extracted with 150 ml methylene chloride, the organic phases washed with H_2O, dried over Na_2SO_4, filtered and evaporated. The amorphous residue is purified chromatographically using 100 g silica gel with methylene chloride/methanol (95 : 5) as eluant to yield the pure title compound: $[\alpha]_D^{20} = -165°$ (c = 1.0 in $CHCl_3$).

The residue at the 8-position has the formula



EXAMPLE 7: Preparation of immunogenic cyclosporin-carrier conjugates of the type defined under 2.1 above: [Process step vi)]

7.1 Conjugate with Chicken γ-Globulin

10 mg of [(N-ε-succinimidooxysuccinyl)-(D)Lys]8-Cyclosporine produced in accordance with the method of example 4 in 0.2 ml dimethylformamide are added to 100 mg chicken γ-globulin in 4 ml $NaHCO_3$ (1.5 % w/v, pH 8.1). The reaction mixture is stirred for ca. 2 hours at room temperature and the obtained conjugate purified by dialysis against phosphate buffered saline.

7.2 Conjugate with Chicken Ovalbumin

10.7 mg [(O-succinimidooxysuccinyl)-Thr]2-Cyclosporine produced in accordance with example 5 and additionally containing 10 % ditritrated material (1 - 2 µCi/mg - obtained analogously to example 5, but using tritrated [Thr]2-Cyclosporine as starting material) in 100 µl dimethylformamide are added with vigorous stirring to 30.45 mg chicken ovalbumin in 2 ml, 1.5 % $NaHCO_3$ buffer (molar excess cylosporin/ovalbumin = 10.68). The reaction mixture is stirred for 2 hours at ambient temperature and the obtained conjugate purified by dialysis 3x against phosphate buffered saline for 18 hours at 4 °C. For the conjugate product

55.7 % of input radioactivity is found bound to ovalbumin, indicating a binding ratio of 5.95 cyclosporin/ovalbumin.

Covalent binding of cyclosporin to ovalbumin was evaluated by acetone precipitation of 3 conjugate aliquots . 39.5 % of radioactivity corresponding to non-covalently bound cyclosporin is determined in the acetonic supernatant, giving a final covalent coupling ratio of 3.6 cyclosporin/ovalbumin. The obtained conjugate was aliquoted and kept at - 20 ° C.

Similar conjugates may be prepared analogously to examples 7.1 and 7.2 above, but employing the product of example 6 as the cyclosporin starting material.

EXAMPLE 8: Preparation of immunogenic cyclosporin-carrier conjugates of the type defined under 2.4/2.6 above: [Process step (x)]

8.1 Conjugate with Guinea pig IgG

Guinea pig IgG (30 mg) are dissolved in 0.1M bicarbonate buffer (pH 9, 0.5 ml) and added to a solution of [(D)Lys]^{8}-Cyclosporine (1 mg) prepared in accordance with the method of example 1, in the same buffer (0.5 ml) and a few drops of 1 % acetic acid are added. Coupling is effected by subsequent addition of 2 portions of N-ethyl-N'-(3-dimethylaminopropyl)carbodiimide hydrochloride (each portion = 100 mg). The reaction product is prepared for injection 24 hours later by multiple dialysis against water followed by lyophilisation.

8.2 Conjugate with Rabbit IgG

[(D)Lys]^8-Cyclosporine (10 mg), prepared in accordance with the method of example 1, is dissolved in 0.1 ml of an ethanolic solution of 6.9 µg (= 0.1 mCi) tritiated [(D)Lys]^8-Cyclosporine. 0.1 ml pyridine and 0.1 ml 35 % formaldehyde are added and the whole is held for 30 mins. at room temperature. 20 mg rabbit IgG in 0.02M phosphate-buffered saline (1 ml) and 0.3 ml pyridine are then added and the reaction allowed to stand for ca. 14 hrs. at room temperature. The obtained conjugate is dialysed against 30 % pyridine, 10 % pyridine and, finally, 0.005M phosphate-buffered saline. The coupling rate of the conjugate is 1:11.4.

EXAMPLE 9: Production of hybridoma cell-lines, producing monoclonal antibodies reactive with Cyclosporine: [Process step viii)]

9.1 Employing the conjugate of example 7.1

a) Immunisation

Female Balb/c mice (20-25 g) each receive 100 µG of the immunogenic conjugate product of example 7.1 in 0.2 ml complete Freund adjuvant, administered by i.p. injection. After 2 weeks a second booster injection comprising 50 µg of the product of example 7.1 emulsified in 0.2 ml complete Freund adjuvant is administered, again by i.p. injection. The presence of antibodies reactive to Cyclosporine in the serum of treated mice is confirmed by regular RIA assay employing tritium labelled Cyclosporine as tracer.

b) Hybridoma Generation

Mice obtained in step a) exhibiting maximum Cyclosporine reactive antibody titres receive a booster injection comprising 20 µg of the product of example 3.2 in saline (0.85 % w/v) administered i.v.. The mice are sacrificed on the 4th. day, and spleen cells, isolated and fused with mouse (Balb/C) myeloma cells in accordance with the methods described by S. Fazekas et al., J. Immunol. Methods, 35, 1 - 21 (1980).

Growing hybridomas are screened for production of antibody reactive to Cyclosporine by regular RIA assay technique employing tritium labelled Cyclosporine as tracer, and exhibiting low cross-reactivity with Cyclosporine 17, again using regular RIA assay technique with tritum labelled Cyclosporine as tracer and Cyclosporine 17 as competitive ligand.

One selected hybridoma line is found to produce a monoclonal antibody reactive with Cyclosporine and having low cross-reactivity with Cyclosporine 17. The antibody is characterised as belonging to the class IgG, subclass IgG_1. The obtained IC_{50} value for reactivity with Cyclosporine in RIA is 6.7 ng/ml, compared with 280 ng/ml for Cyclosporine 17. Cross-reactivity with Cyclosporine 17 is thus of the order of 2 % only. Determined affinity constant with respect to Cyclosporine is of the order of 10^{-9} mol/litre.

It will be appreciated that by application of the techniques of the present invention as generally taught herein, in particular the employment of cyclosporins having an activated coupling group, e.g. as hereinbefore defined under any one of 1.1 to 1.11, 1.14 or 1.15 (formulae IIa, IIb, IIc or IId) for the preparation of immunogenic conjugates, and proceeding e.g. analogously to the general methods of this example, hybridoma lines/monoclonal antibodies may readily be prepared which, though not identical with the specific product hybridoma line/monoclonal antibody of this example, will meet the same essential criteria, e.g. exhibit equivalent or even improved characteristics to those described above. This will be apparent from results evidenced in the following example.

9.2 Employing the conjugate of example 7.2

a) Immunisation

Mice (Balb/c) each receive 100 µg of the immunogenic conjugate product of example 7.2 in 200 µl phosphate buffered saline/Freund adjuvent (1 : 1). The first administration (complete Freund adjuvent) is effected s.c. in the hind foot pad, near the tail and near the neck. After three weeks, second and third administrations follow (incomplete Freund adjuvent) effected s.c. on the back and i.m. in the hind legs respectively. Blood samples are collected 1 week after both the 2nd and 3rd administrations.

Mice are selected for further use on the basis of the following measured antisera criteria:

1. Titre in liquid phase RIA and in ELISA;

2. Apparent isotype distribution (IgG_1 only or $IgG_{1+2a+2b}$ in ELISA);

3. Relative avidity in ELISA;

4. Capacity to discriminate between Cyclosporine and Cyclosporine 17 and Cyclosporine 18 in competitive ELISA.

Selected mice are given booster injections on days -3, -2 and -1 prior to fusion using 100 µg of the immunogenic conjugate product of example 7.2 in 200 µl, 9 % NaCl, by i.p. (50 %) and i.v. (50 %) injection on day -3, and i.p. (100 %) on days -2 and -1.

b) Hybridoma generation

2.5 x 10^{-7} or 5 x 10^{-7} spleen cells from each mouse are fused with 5 x 10^{-7} mouse (Balb/c) myeloma cells using PEG 4000 and distributed into 24 x 24 wells.

Culture supernatants are screened in ELISA for the presence of antibodies recognising $[Thr]^2$-Cyclosporine coupled to bovine serum albumin (prepared analogously to example 7.2) and/or $[(D)Lys]^8$-Cyclosporine coupled to bovine serum albumine (prepared analogously to example 7.1) in preference to free bovine serum albumin as negative control. Selected IgG producing hybridoma lines are cloned to guarantee monoclonality.

Ability of monoclonal antibodies produced by hybridoma lines obtained, to distinguish/discriminate between (a) Cyclosporine and (b) Cyclosporine 17 and Cyclosporine 18 is tested in a competition format of indirect ELISA as described by Quesniaux et al., Immunology Letters, 9, 99 - 104, (1985), in a variety of buffer systems including: phosphate buffered saline at pH 7.5, with and without 0.03 % Tween 20; and Tris at pH 7.5, with 0.03 % Tween and without NaCl. Optimal conditions for discrimination are generally observed in phosphate buffered saline at pH 7.5, with 140mM NaCl and 0.03 % Tween 20. Of 9 clone lines examined 7 produce monoclonal antibodies discriminating between (a) Cyclosporine and (b) Cyclosporines 17 and 18. For 6 the IC_{50} ratio of Cyclosporine 17 compared to Cyclosporine is ca. 35x or greater.

EXAMPLE 10: Production of regular polyclonal antisera reactive with Cyclosporine [Process step xi)]

Sheep are immunised by hind limb, intramuscular injections 10x at intervals of approx. 14 days. The injections comprise a lyophylisate of the immunogenic protein conjugate product of example 8.1 (3 mg), Alugel S (0.2 ml) and Freund adjuvant (0.6 ml). The final titre obtained against tritiated Cyclosporine is 1/100,000 as measured by RIA.

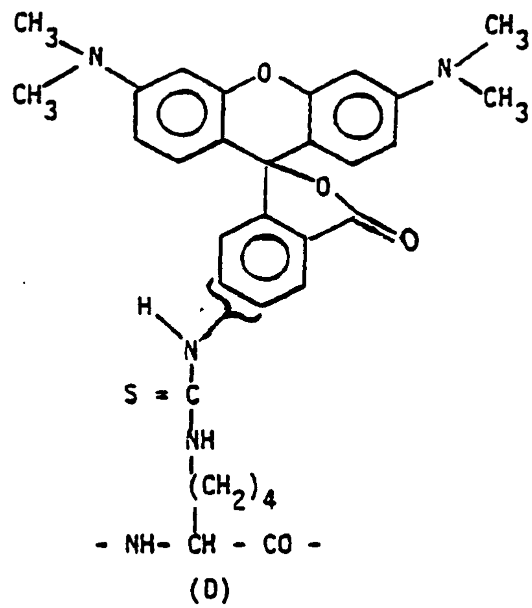
Polyclonal antisera recovered are found to exhibit improved discrimination between Cyclosporine and its metabolite Cyclosporine 17 as compared with polyclonal antisera obtained using [Thr]^2-Cyclosporine-IgG conjugates known in the art, e.g. as employed in current Cyclosporine RIA assay kits. Thus for antisera obtained in accordance with the present example, cross-reactivity with Cyclosporine 17 is of the order of ca. 18 % as compared with ca. 42.0 % for known polyclonal antisera.

EXAMPLE 11: Preparation of labelled derivatives of cyclosporins as defined under 5.1 above: [Process step ix)]

11.1: Preparation of [N- -TRITC-(D)Lys]^8-Cyclosporine

[(D)Lys]^8-Cyclosporine (15 mg) produced in accordance with example 1 are dissolved in methylene chloride (2 ml). Rhodamine isothiocyanate (TRITC) (5.3 mg) is added and the reaction mixture allowed to stand at -7°C for 17 hours. The intensively-red-coloured solution is directly chromatographed on Sephadex LH20 (20 g) with methylene chloride and 0.5 % methanol. Fractions are collected in 10 ml portions.

The title compound is recovered as an oil from fractions 5 to 7 and 10 to 14: UV absorption: 300 nm/fluorescence emission: 540 nm. The residue at the 8-position has the structure:



11.2: Preparation of [N-ε-Dansyl-(D)Lys]^8-Cyclosporine

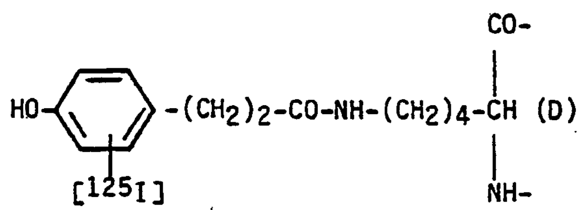
[(D)Lys]^8-Cyclosporine (232 mg) produced in accordance with example 1 is dissolved in chloroform (15 ml). Ethyl diisopropylamine (7.3 mg) and dansyl chloride (99.5 mg) are added and the reaction mixture is stirred for 2 hours. The product is chromatographed directly on Sephadex LH20 (100 g) with methylene chloride and 0.5 % methanol. Fractions are collected in 10 ml portions.

The collected fractions are evaporated and the resulting product re-chromatographed using silica gel (0.06 - 0.20 mm) (100 g) with methylene chloride and 5 % methanol. Fractions are collected in 15 ml portions.

Fractions 28-44 yield pure product: $[\alpha]_D^{20} = -183.8°$, c = 1.08 in $CHCl_3$.

11.3: Preparation of ^{125}Iodinated Derivative of [(D)Lys]^8-Cyclosporine

Title compound is prepared analogously to the methods described by Bolton and Hunter [Biochem. J. 133, 529 (1973)] by attachment of a p-OH-phenylpropionyl residue to the N-ε-atom of the residue at the 8-position of [(D)Lys]^8-Cyclosporine prepared in accordance with example 1. The ^{125}I label is carried in the phenyl ring of the p-OH-phenylpropionyl residue



Purification is effected by HPLC on a 4x 250 column of RP18 with a linear gradient and using 10 - 30 % n-propanol/0.2 % trifluoroacetic acid in 5 % acetic acid/0.2 % trifluoroacetic acid as liquid phase.

CLAIMS:

1. A monoclonal antibody capable of distinguishing between a cyclosporin and a metabolite thereof.

2. A hybridoma cell line producing a monoclonal antibody according to claim 1.

3. A cyclosporin

   a) having an α-amino acid residue bearing an activated coupling group;

   b) having an amino substituted (D)α-amino acid residue at the 8-position the amino substituent being in free or protected form; or

   c) having an acylamino substituted (D)α-amino acid residue at the 8-position, wherein the acylamino substituent is substituted in the acyl moiety thereof by a free carboxy group.

4. An immunogenic conjugate comprising a carrier coupled to a cyclosporin by the agency of an activated coupling group, e.g. an immunogenic conjugate comprising a carrier coupled to a cyclosporin according to claim 3 part a).

5. An immunogenic conjugate capable of use in the production of a monoclonal antibody according to claim 1.

6. An immunogenic conjugate comprising a carrier coupled to a cyclosporin via the α-amino acid residue at position 5-, 6-, 7- or 8- of said cyclosporin.

7. Antibody reactive with a cyclosporin, including polyclonal antiserum reactive with a cyclosporin, generated in response to an immunogenic conjugate according to claim 6.

8. A labelled derivative of a cyclosporin wherein the residue at the 8-position is -(D)Lys-.

9. An immunoassay kit or system for cyclosporin assay comprising

   A) antibody or antiserum according to claim 6; or

   B) a labelled derivative of a cyclosporin according to claim 10

   as kit or system component.

10. Each and every novel process, method, product, manner of manufacture or other feature hereinbefore described or any combination thereof.

INTERNATIONAL SEARCH REPORT

International Application No PCT/EP 85/00501

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) [6]

According to International Patent Classification (IPC) or to both National Classification and IPC

IPC[4]: C 07 K 15/00; C 12 P 21/00; C 12 N 5/00; C 07 K 7/64; G 01 N 33/53 // (C 12 P 21/00; C 12 R 1:91)

II. FIELDS SEARCHED

Minimum Documentation Searched [7]

Classification System	Classification Symbols
IPC[4]	C 12 P C 07 K G 01 N C 12 N

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched [8]

III. DOCUMENTS CONSIDERED TO BE RELEVANT [9]

Category *	Citation of Document, [11] with indication, where appropriate, of the relevant passages [12]	Relevant to Claim No. [13]
Y	EP, A, 0044441 (SCRIPPS-MILES INC.) 27 January 1982, see claims 1,2,4-7; page 3, line 33 - page 4, line 1; page 5, lines 18-25 --	1,2
Y	Chemical Abstracts, volume 95, no. 23, 7 December 1981, Columbus, Ohio, (US) P. Donatsch et al.:"A radioimmunoassay to measure cyclosporin A in plasma and serum samples", see pages 9-10, abstract no. 197042f & J. Immunoassay 1981, 2(1), 19.32 --	1,2
A	Chemical Abstracts, volume 100, no. 17, 23 April 1984, Columbus, Ohio, (US) G. Maurer et al.:"Disposition of cyclosporine in several animal species and man. I. Structural elucidation of its metabolites", see page 9, abstract no. 132001u & Drug Metab. Disps. 1984, 12(1), 120-6 (cited in the application) --	

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* Special categories of cited documents: [10]

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
27th February 1986	25 MARS 1986
International Searching Authority	**Signature of Authorized Officer**
EUROPEAN PATENT OFFICE	M. VAN MOL

International Application No. PCT/EP 85/00501

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)

Category *	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No
P,X	Chemical Abstracts, volume 102, no. 23, 10 June 1985, Columbus, Ohio, (US) V. Quesniaux et al.:"An enzyme immuno-assay for the screening of monoclonal antibodies to cyclosporin", see page 452, abstract no. 202207h & Immunol. Lett. 1985, 9(2-3), 99-104 (cited in the application)	1,2

International Application No. PCT/EP 85/00501

FURTHER INFORMATION CONTINUED FROM THE SECOND SHEET

V. [X] OBSERVATIONS WHERE CERTAIN CLAIMS WERE FOUND UNSEARCHABLE [1]

This international search report has not been established in respect of certain claims under Article 17(2) (a) for the following reasons:

1. [X] Claim numbers °°)... because they ~~xxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxx~~

°°) 5, 9 and 10 represent matter which is vague and as such is difficult to search

2. [ ] Claim numbers ...., because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. [ ] Claim numbers........., because they are dependent claims and are not drafted in accordance with the second and third sentences of PCT Rule 6.4(a).

VI. [X] OBSERVATIONS WHERE UNITY OF INVENTION IS LACKING [2]

This International Searching Authority found multiple inventions in this international application as follows:

- claims 1,2 : Monoclonal antibody and hybridoma cell line producing it
- claim 3 : Cyclosporins having an activated coupling group
- claims 4,5,6: Immunogenic conjugates

-/--

1. [ ] As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims of the international application.
2. [ ] As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims of the international application for which fees were paid, specifically claims:
3. [X] No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claim numbers: 1 and 2.
4. [ ] As all searchable claims could be searched without effort justifying an additional fee, the International Searching Authority did not invite payment of any additional fee.

Remark on Protest

[ ] The additional search fees were accompanied by applicant's protest.

[ ] No protest accompanied the payment of additional search fees.

FURTHER INFORMATION CONTINUED FROM PCT/ISA/210 (supplemental sheet (2))

- claim 7: Antibody reactive with a cyclosporin
- claim 8: Labelled derivative of a cyclosporin
- claim 9: Cyclosporin immunoassay kit or system

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ANNEX TO THE INTERNATIONAL SEARCH REPORT ON

INTERNATIONAL APPLICATION NO. PCT/EP 85/00501 (SA 10999)

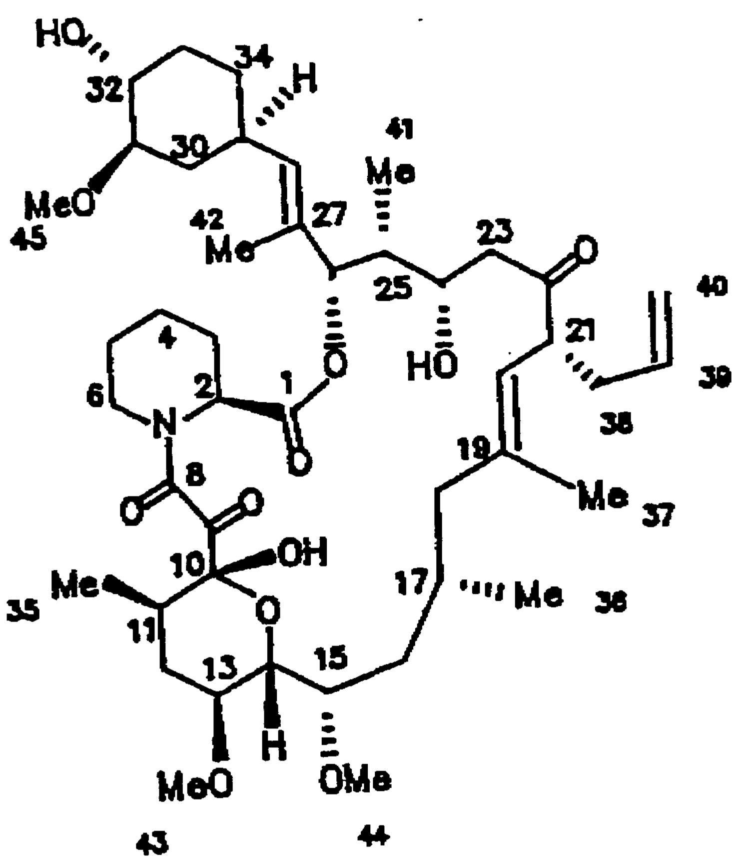
This Annex lists the patent family members relating to the patent documents cited in the above-mentioned international search report. The members are as contained in the European Patent Office EDP file on 17/03/86

The European Patent Office is in no way liable for these particulars which are merely given for the purpose of information.

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
EP-A- 0044441	27/01/82	JP-A- 57053411	30/03/82

For more details about this annex :
see Official Journal of the European Patent Office, No. 12/82

FIGURE 1



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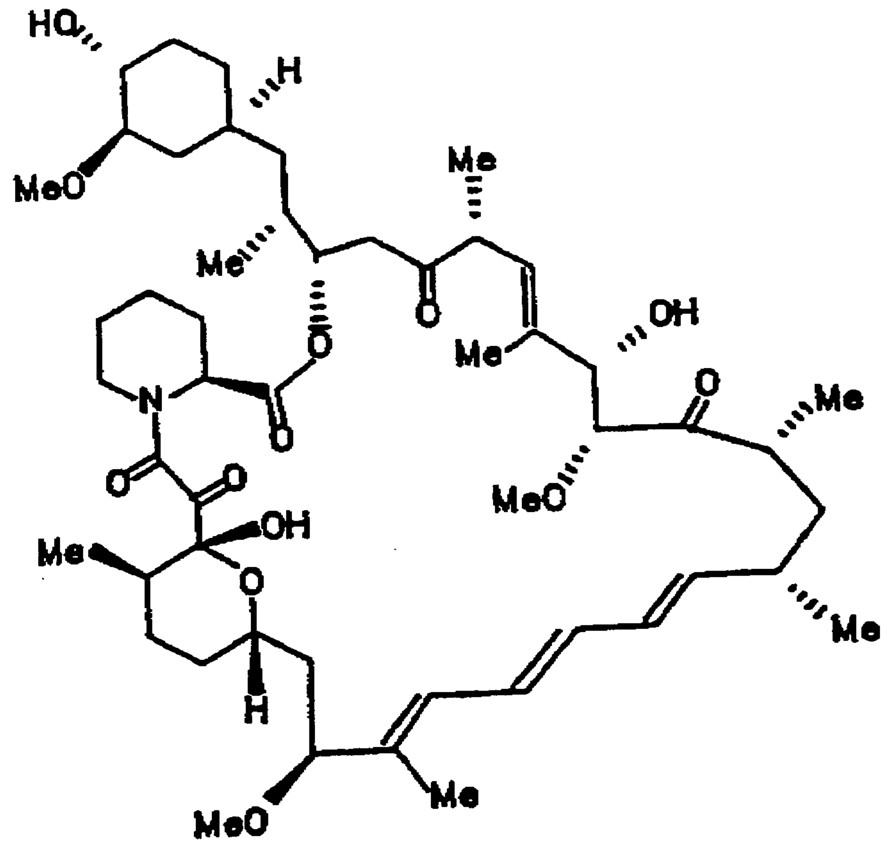

Rapamycin

PORTIONS OF THE MOLECULES THAT ARE THE SAME